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To cite this article: Dukanya Dukanya , Rashmi Sivakumar , Narasimha M. Beeraka , Hosadurga Kumar Keerthy , Keerthikumara Venkatesha , Shreeja Basappa , Vladimir N. Nikolenko , Kanchugarakoppal S Rangappa , Andreas Bender & Basappa Basappa (29 Mar 2026): Nano-TiO₂ Catalyst-Based Method Development to Synthesize Pyrano-Chromenes Targeting TFF3 in Breast Cancer, Polycyclic Aromatic Compounds, DOI: [10.1080/10406638.2026.2638969](https://doi.org/10.1080/10406638.2026.2638969)

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Published online: 29 Mar 2026.



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Nano-TiO₂ Catalyst-Based Method Development to Synthesize Pyrano-Chromenes Targeting TFF3 in Breast Cancer

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ABSTRACT

Trefoil Factor 3 (TFF3) is an oncogenic protein implicated in breast cancer progression through regulation of PI3K-AKT, MAPK, STAT3, and NF- κ B signaling pathways. Targeting TFF3 therefore represents a promising therapeutic strategy. In this study, chromene-3-carbonitrile derivatives were synthesized using nano-TiO₂ as an efficient catalyst and evaluated for their anticancer potential against TFF3 in MCF-7 breast cancer cells through combined *in vitro* and *in silico* approaches. Thermodynamic feasibility of the synthetic process was assessed using free energy calculations. Cytotoxicity of the synthesized compounds was determined in MCF-7 cells, while molecular docking studies were performed to investigate binding interactions with TFF3. Protein expression analysis was performed using western blotting to confirm TFF3 inhibition. Several derivatives demonstrated significant cytotoxic activity, with lead compounds F8, F10, F12, and F13 exhibiting IC₅₀ values of 0.751, 1.70, 0.68, and 0.52 μ M, respectively. Docking studies revealed strong binding affinity and favorable interactions with TFF3 and energy profiling supported the thermodynamic viability of synthetic route. Western blot results further confirmed downregulation of TFF3 expression. Overall, these findings identify chromene-3-carbonitrile derivatives as potent TFF3-targeting agents with potential therapeutic relevance for breast cancer.

HIGHLIGHTS

- Successful synthesis of chromene-3-carbonitrile derivatives using nano-TiO₂ as a catalyst.
- *In vitro* cytotoxicity assays demonstrated significant anticancer effects of the synthesized compounds.
- Molecular docking confirmed strong interactions of lead compounds with TFF3.
- Western blot analysis validated the inhibitory effects of the compounds on TFF3 expression.
- Potential development of chromene-3-carbonitrile derivatives as targeted therapies for MCF-7 cells.

NOVELTY STATEMENT

This study introduces a novel approach to targeting TFF3 in breast cancer by synthesizing chromene-3-carbonitrile derivatives using nano-TiO₂ as a catalyst. The combined *in vitro* and *in silico* analyses provide new insights into the potential of these com-

ARTICLE HISTORY

Received 22 November 2025
Accepted 25 February 2026

KEYWORDS

Trefoil factor 3; chromene-3-carbonitrile; breast cancer; Nano-TiO₂; cytotoxicity

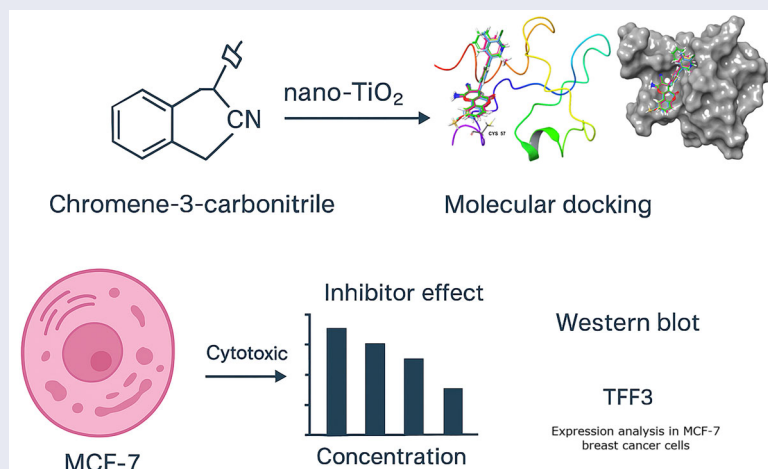
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 Supplemental data for this article can be accessed online at <https://doi.org/10.1080/10406638.2026.2638969>.

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pounds as therapeutic agents for breast carcinomas, highlighting their significance in drug development.

GRAPHICAL ABSTRACT



Introduction

Breast cancer (BC) is the most prevalent malignancy among women and remains the leading cause of cancer-related mortality globally.¹ BC encompasses various types, which are classified based on their tissue of origin and molecular characteristics.² Microarray gene expression analyses of breast tumors have delineated four principal molecular subtypes: Luminal, Normal Breast-like, HER2-enriched and Basal-like/Triple-negative BC.²

Trefoil factor (TFF) peptides, a family of three small regulatory proteins, include TFF3, which is expressed in both normal and cancerous breast tissues at the protein and mRNA levels.^{3,4} The expression of TFF3 varies in invasive breast cancers.⁵ TFF3 is characterized by the trefoil domain, a three-looped structure stabilized by three disulfide bonds.^{6,7} TFF3 is a small secreted peptide belonging to the trefoil family, originally characterized for its role in mucosal protection and epithelial restitution. However, mounting evidence supports its oncogenic functions in various cancers, particularly breast carcinoma, where TFF3 expression has been linked to increased proliferation, survival, invasion and therapy resistance.^{8,9} Early studies demonstrated that upregulated TFF3 expression in MCF-7 cells enhanced survival and invasiveness, while depletion of TFF3 reduced oncogenicity and improved sensitivity to anti-estrogens, demonstrating a functional role in tumor progression and drug resistance.^{8,9} Research has extensively explored TFF3's role in tumor progression, revealing its aberrant expression in malignant cells from various cancers, including those of the liver, colon, breast, endometrium, prostate, cervix, stomach, lung, and brain.^{4,5,10–17} Overexpression of TFF3 is associated with chemoresistance, reduced relapse-free survival, and poorer overall survival in cancer patients.¹⁸ It has also been linked to an increased risk of pituitary cancer through activation of mitochondrial apoptotic pathways.^{19,20} Inhibiting TFF3 has been shown to reduce invasion, migration, and tumor formation in papillary thyroid cancer cells by downregulating AKT phosphorylation. TFF3's oncogenic function involves regulating signaling pathways such as PI3K-AKT, MAPK, STAT3 and NF- κ B.^{4,5,8,10–17,21–25} Additionally, ectopic expression of TFF3 in colorectal cancer cells has been found to enhance stem cell-like properties, resistance to 5-Fluorouracil and carcinogenesis.¹²

Several small molecules with heterocyclic structures are synthesized as inhibitors targeting breast cancer cells and their signaling pathways.²⁶ Notable FDA-approved pyridine derivatives include Neratinib for early-stage HER2-positive BC,²⁷ Ribociclib for HR+/HER2– advanced BC, and Palbociclib for HR+/HER2– metastatic BC and other drugs such as coumarins and their derivatives have significant roles in cancer research.^{28–32} Drugs such as Novobiocin, Warfarin, and Irosustat are

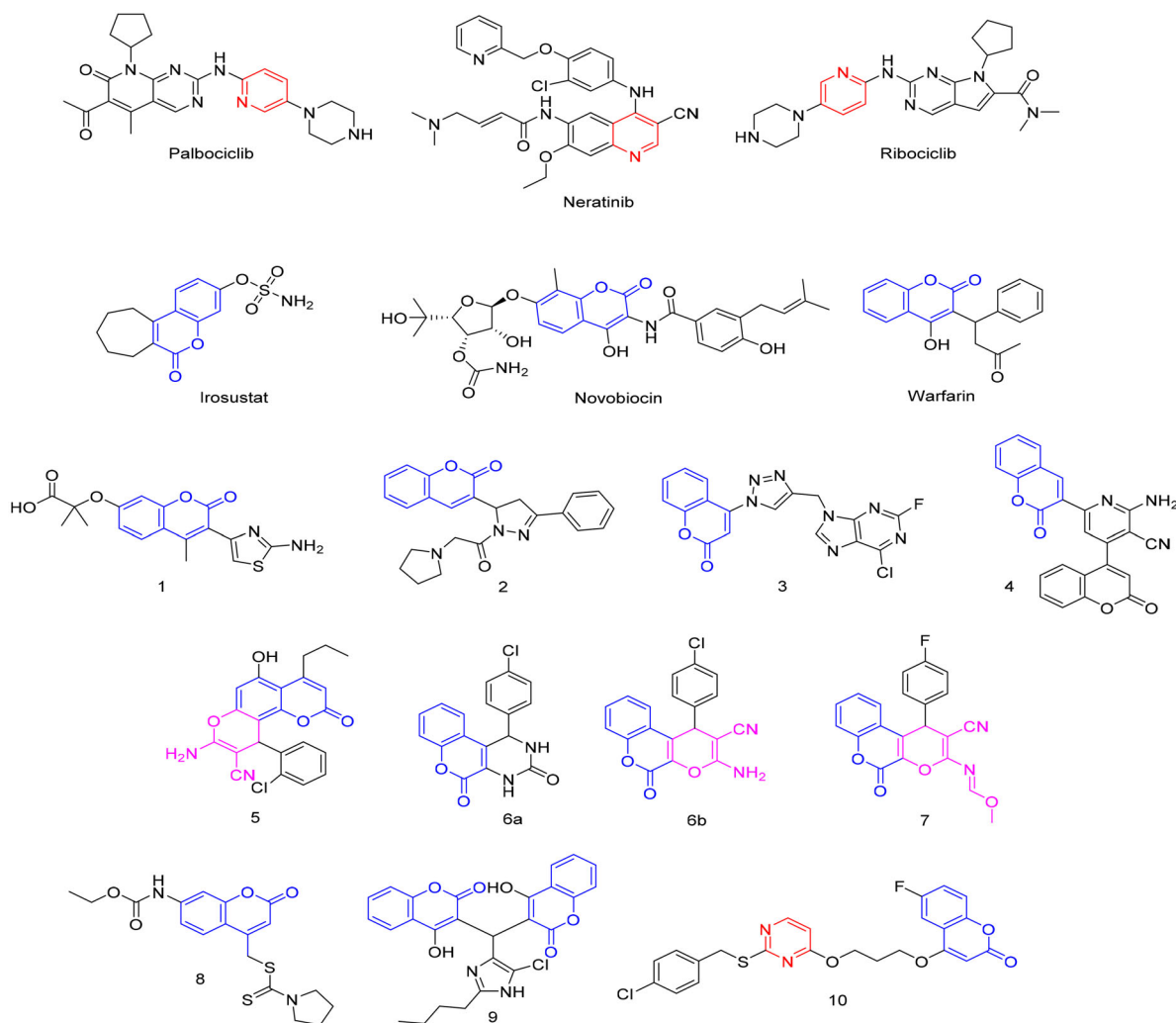


Figure 1. Representation of pharmacological scaffold diversity: exploration of drug and anticancer agent structures featuring pyridine (red), coumarin (blue), and pyrane (pink) motifs.

among the coumarin-containing therapeutics (Figure 1).³² Various coumarin derivatives have shown potent anticancer activity *in vitro* against breast cancer cell lines MCF-7 and MDA-231. Thiazole-substituted coumarins,³³ pyrazoline-substituted coumarins³⁴ and triazole-substituted coumarins³⁵ are significant examples. Other coumarin-based compounds, such as coumarin-pyridine-amino-carbonitriles,³⁶ chromene-9-carbonitriles³⁷ and chromene-pyrimidinediones,³⁸ have demonstrated efficacy against different cancer cell lines. Our research has identified AMPC (2-amino-4-(4-(6-fluoro-5-methylpyridin-3-yl)phenyl)-5-oxo-4H,5H-pyrano[3,2-c]chromene-3-carbonitrile) derivatives as covalent, reversible inhibitors of TFF3. AMPC disrupts TFF3 homodimerization by interacting with the CYS57-CYS57 residue, promoting monomer formation in MCF-7 cells, leading to apoptosis and decreased cell viability through caspase 3/7 activation.³⁹ Further studies revealed that AMPC enhances the efficacy of 5-Fluorouracil in CMS4 subtype colorectal cancer cells, demonstrating a synergistic reduction in cancer cell proliferation and survival.¹²

Additionally, AMPC increases the efficacy of doxorubicin in ER+ BC cells, exhibiting strong anti-cancer activity in doxorubicin-resistant ER+ BC cells and xenograft models.⁴⁰ In lung adenocarcinoma, AMPC inhibited the growth of H1975 and H1299 cells, and its combination with MEK1/2 inhibitors or EGFR TKIs indicated potential therapeutic strategies for TKI-resistant cancers.¹⁷ AMPC also significantly inhibited pancreatic ductal adenocarcinoma (PDAC) cell proliferation *in vitro* and *in vivo*, particularly when combined with WNT inhibitors.⁴¹ Beyond survival and migration regulation, TFF3 has been associated with activation of key signaling cascades, including PI3K-AKT, MAPK/ERK, and STAT3 pathways,⁹ which are essential mediators of cancer cell proliferation, apoptosis evasion, and

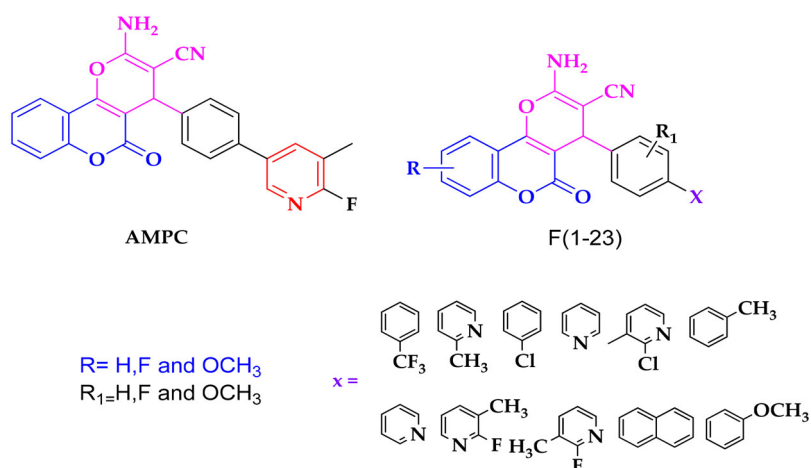


Figure 2. Previously reported TFF3 inhibitor AMPC and its derivatives F(1–23): structural variants and pharmacological implications.

metastatic potential. For instance, in colorectal cancer models, TFF3 knockdown reduced PD-L1 expression *via* AKT/mTOR signaling, improving antitumor immune infiltration.⁹ Given these multifaceted roles, TFF3 has emerged as a viable therapeutic target in hormone-responsive and resistant breast malignancies and other tumors, making the identification of small molecule TFF3 antagonists a priority in preclinical oncology research.⁴²

Chromene-3-carbonitrile derivatives heterocyclic frameworks with diverse substitution patterns have attracted attention in medicinal chemistry owing to their broad pharmacological profiles including anti-cancer activity. Recent synthetic approaches have produced libraries of 4H-chromene-3-carbonitrile derivatives showing activity against tyrosinase and various cancer cell lines, with structural modifications influencing potency and selectivity.⁴³ Moreover, multicomponent reactions and nano-catalytic strategies using advanced catalysts such as nano-TiO₂ have enabled efficient formation of chromene cores under mild conditions with high yields and favorable sustainability profiles.⁴⁴

Recent advances in nanocatalyst development, such as TiO₂ nanoparticles supported on magnetic substrates, have enabled efficient one-pot synthesis of biologically important heterocycles with high yields and reusability. TiO₂-based nanocatalytic systems have been successfully applied to pyrano[3,2-c]chromene synthesis, demonstrating improved reaction efficiency and selectivity under eco-friendly conditions.^{44–47} Furthermore, tailored nanocatalysts development significantly enhance the efficiency, sustainability and selectivity of chromene and related heterocycle synthesis. Magnetic Fe₃O₄-based hybrid catalysts have been successfully applied for one-pot chromene synthesis with high yields and facile recovery, demonstrating their potential in green synthetic chemistry.⁴⁸

Despite progress in chromene medicinal chemistry and TFF3 biology, few studies have explored chromene-3-carbonitrile derivatives as direct inhibitors of TFF3, particularly within the context of breast cancer models such as MCF-7 cells. Bridging this gap could yield promising leads for targeted TFF3 suppression, offering both cytotoxic and signaling-modulatory benefits.⁴⁹ Collectively, these findings highlight TFF3 as a promising biomarker and therapeutic target in cancer treatment. Building on our previous research, in this study, we report the synthesis of chromene-3-carbonitrile derivatives using nano-TiO₂ particles and their evaluation against MCF-7 cells, focusing on their potency in inhibiting TFF3 (Figure 2).

Objective and novelty of the study

Although TFF3 is increasingly recognized as an oncogenic driver that promotes proliferation, resistance, and pro-survival signaling (PI3K-AKT, MAPK, STAT3) in breast and other cancers, relatively few small-molecule inhibitors of TFF3^{39,42,50,51} have been reported and none have systematically combined target-directed design with green, nano-catalyzed synthesis of chromene scaffolds. Recent reports demonstrate feasibility of pharmacologic TFF3 inhibition to overcome endocrine resistance, but examples

remain limited and medicinal scaffolds suited for TFF3 engagement are lacking. Therefore, we designed and synthesized a focused library of chromene-3-carbonitrile derivatives using nano-TiO₂ catalysis, evaluated the thermodynamic feasibility of their synthesis, and assessed their anticancer activity, TFF3 binding (molecular docking) and modulation of TFF3 protein levels in MCF-7 cells.

The primary objective of this study was to synthesize a series of chromene-3-carbonitrile derivatives using nano-TiO₂ as a catalyst and evaluate their potential as inhibitors of TFF3 in MCF-7 breast cancer cells through integrated *in vitro* cytotoxic assays, thermodynamic analysis, molecular docking, and protein expression studies. While chromene-3-carbonitriles have been explored for enzymatic inhibition and cytotoxicity, this work is novel in several critical respects: (1) It applies nano-TiO₂ catalysis in the synthesis of chromene-3-carbonitrile derivatives specifically tailored for anticancer evaluation, offering a greener, efficient synthetic route that leverages the surface reactivity and recyclability of nanostructured TiO₂.⁴⁴ (2) It is among the first studies to target TFF3 with small molecule chromene derivatives in breast cancer cells, extending chromene medicinal chemistry into the direct modulation of an oncogenic signaling mediator. (3) The integration of thermodynamic feasibility analyses, *in vitro* cytotoxicity, molecular docking to TFF3 and Western blot assessment of TFF3 expression provides a comprehensive therapeutic assessment pipeline. This combination of methodologies is seldom applied in chromene-based antitumor compound evaluations.⁴³ (4) The identification of lead compounds with sub-micromolar IC₅₀ values against MCF-7 cells that also exhibit strong predicted binding to TFF3 highlights their potential as novel chemical scaffolds for targeted breast cancer therapy.

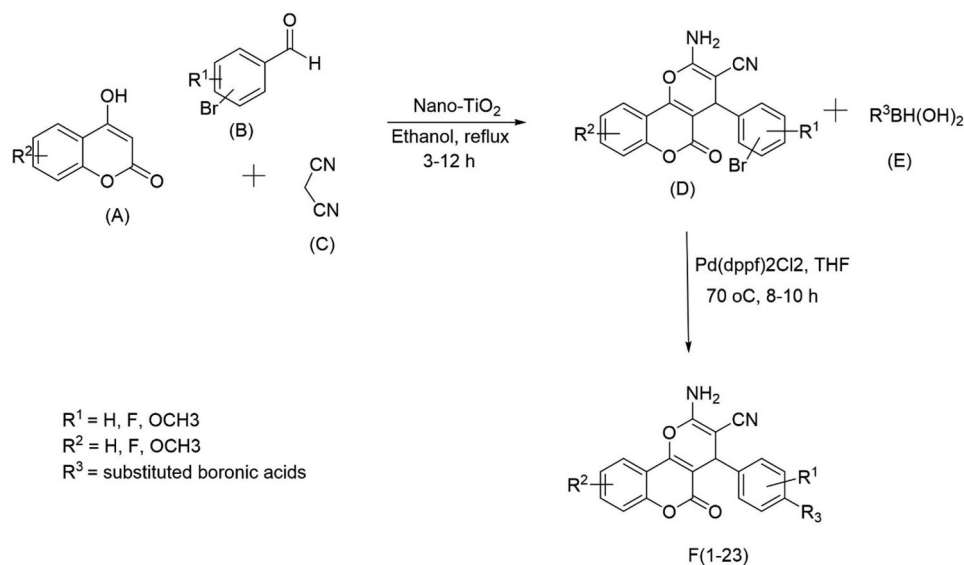
Results

Chemical synthesis

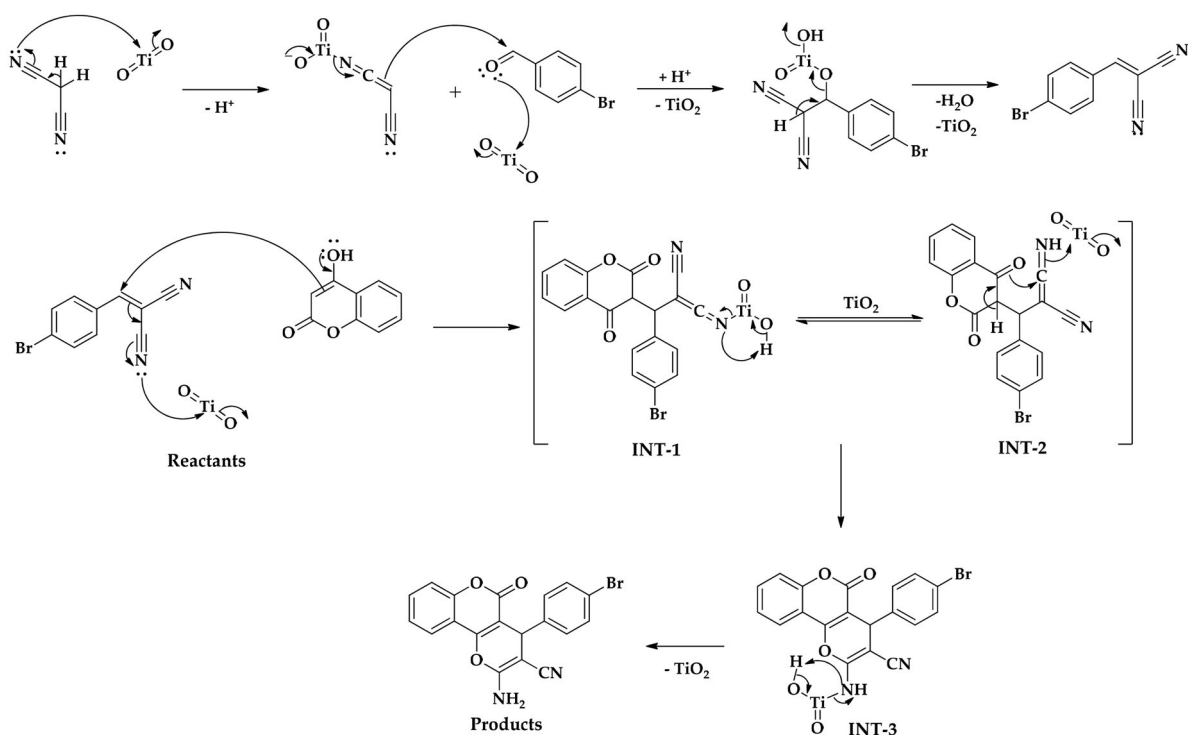
Chromene-3-carbonitriles are a class of tricyclic heterocycles exhibiting significant potential as small molecule inhibitors in anticancer therapies.^{9,39,42,43,49–52} Traditionally, these compounds have been synthesized using a three-component reaction involving aldehydes, malononitrile, and 4-hydroxycoumarin, catalyzed by TBAB under aqueous or solvent-free conditions.⁴⁴ Other reported protocols include the use of catalysts such as DAHP/S-proline, pyridine, DABCO, and CuO nanoparticles, as well as alternative energy inputs like microwave irradiation and electrochemical activation. Nanocatalysts such as TiO₂ and Fe₃O₄-based composites have been reported to facilitate chromene formation through multi-component reactions, offering advantages in reaction time and catalyst recovery.^{47,53,54} Photocatalytic TiO₂-based composites such as TiO₂/ZnWO₃ and TiO₂/CuWO₄ have been shown to promote chromene derivative formation under mild conditions, demonstrating the versatility and catalytic prowess of TiO₂ nanocomposites in heterocyclic synthesis.^{55,56}

In contrast, our Nano-TiO₂-based synthetic approach offers a distinctive advantage by combining high catalytic efficiency with environmental growth. The method proceeds under mild reaction conditions in ethanol, affording excellent yields in shorter reaction times. Furthermore, the use of Nano-TiO₂ not only enhances product selectivity but also aligns well with green chemistry principles due to its reusability and nontoxicity. This novel protocol thus represents a sustainable and operationally simple route for the efficient synthesis of bioactive chromene-3-carbonitriles derivatives.

In the present study, a series of novel 2-aminopyrano[3,2-c]chromene-3-carbonitrile derivatives were synthesized *via* a three-component reaction of 4-hydroxycoumarin, 6-fluoro-4-hydroxy-2H-chromen-2-one or 4-hydroxy-7-methoxy-2H-chromen-2-one (A) with various substituted bromobenzaldehydes (B) and malononitrile (C) in ethanol, using TiO₂ nanoparticles as an efficient catalyst. This reaction furnished the key intermediates, 2-amino-4-(3-substitutedphenyl)-5-oxo-4,5-dihydropyrano[3,2-c]chromene-3-carbonitriles (D), in good yields. Subsequent Suzuki–Miyaura cross-coupling reactions of these intermediates with substituted boronic acids (E) were performed in a water–THF (1:4) system using Pd(dppf)₂Cl₂ as the catalyst and K₂CO₃ as the base, affording the final compounds F(1–23) as shown in Table 1 and illustrated in Scheme 1 and 2.



Scheme 1. Synthetic route for the preparation of chromene-3-carbonitriles: Key steps and structural modifications.



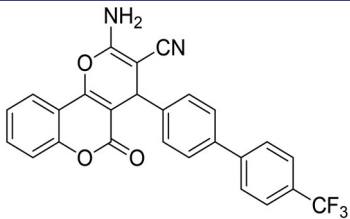
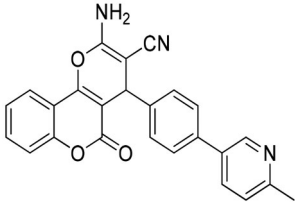
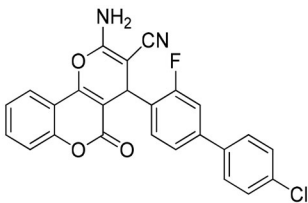
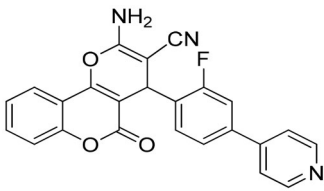
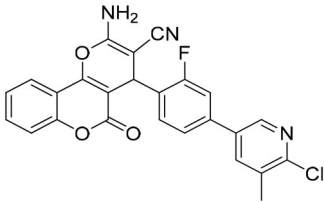
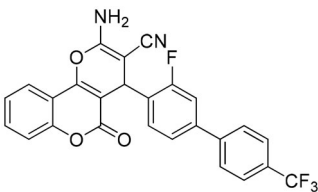
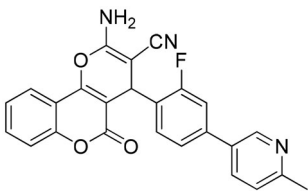
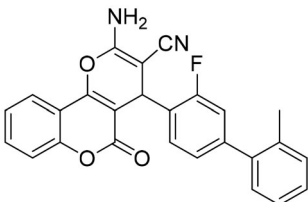
Scheme 2. Proposed reaction mechanism for the formation of chromene-3-carbonitriles.

Gibb's free energy (ΔG) calculations

The molecular geometries of the reactants, intermediates and products involved in the reaction process were rigorously validated using fundamental harmonic vibrational analysis at the same computational level. To elucidate the reaction mechanism, Gibbs free energy calculations were employed to assess the stability of Titanium complexes' intermediates.

Initially, Titanium dioxide (TiO_2) reacts with Bromo benzylidene malononitrile and a hydroxy group, forming an immediate Ti-N bond to produce intermediate 1, stabilized with an energy (ΔE) of 7.185 kcal/mol. Subsequent dissociation of TiO_2 from intermediate 1 leads to the formation of

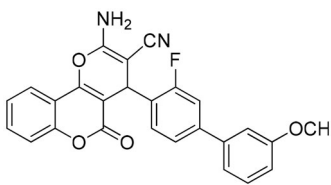
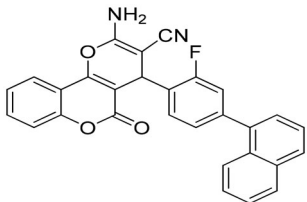
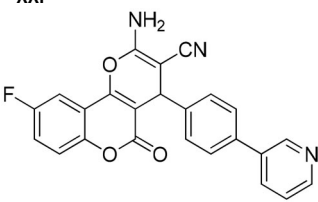
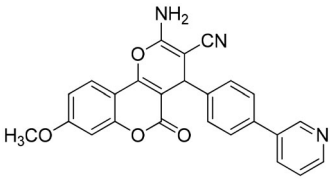
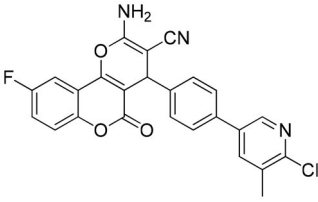
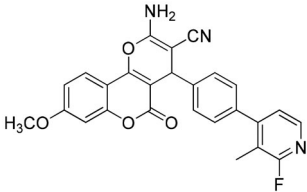
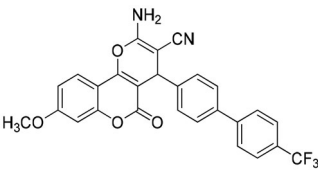
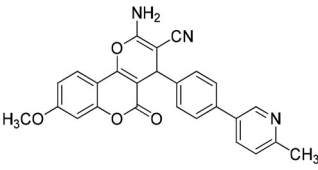
Table 1. Newly synthesized chromene-3-carbonitriles and their cytotoxicity profiles against MCF-7 human breast cancer cells including IC₅₀ values.

Entry	Structure	IC ₅₀ (μM)	TON	TOF (hours)
F1		2.54	445 × 10 ⁴	445 × 10 ⁴ /6
F2		43.80	450 × 10 ⁴	450 × 10 ⁴ /6
F3		3.95	445 × 10 ⁴	450 × 10 ⁴ /8
F4		2.89	465 × 10 ⁴	465 × 10 ⁴ /8
F5		1.20	440 × 10 ⁴	465 × 10 ⁴ /8
F6		1.84	420 × 10 ⁴	465 × 10 ⁴ /8
F7		3.25	430 × 10 ⁴	465 × 10 ⁴ /8
F8		0.751	430 × 10 ⁴	465 × 10 ⁴ /6

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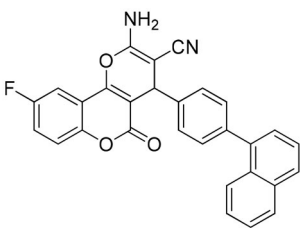
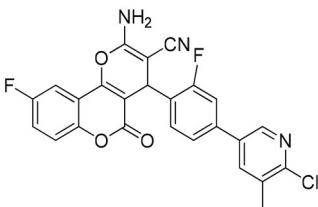
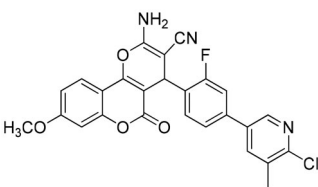
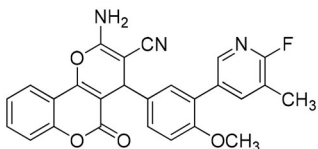
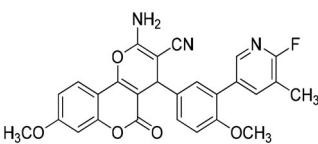
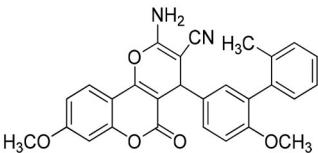
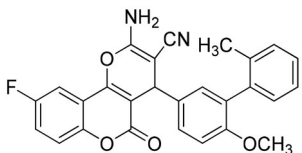
(continued)

Table 1. Continued.

Entry	Structure	IC ₅₀ (μM)	TON	TOF (hours)
F9		1.06	410 × 10 ⁴	465 × 10 ⁴ /8
F10		1.70	450 × 10 ⁴	465 × 10 ⁴ /8
F11	XXI 	6.84	450 × 10 ⁴	465 × 10 ⁴ /3.5
F12		0.68	455 × 10 ⁴	465 × 10 ⁴ /9
F13	XXIII 	0.52	420 × 10 ⁴	465 × 10 ⁴ /3.5
F14	XXVII 	7.376	405 × 10 ⁴	465 × 10 ⁴ /9
F15		2.447	455 × 10 ⁴	465 × 10 ⁴ /9
F16		77.62	410 × 10 ⁴	465 × 10 ⁴ /9

(continued)

Table 1. Continued.

Entry	Structure	IC ₅₀ (μM)	TON	TOF (hours)
F17		22.36	455×10^4	$465 \times 10^4/3.5$
F18		32.52	465×10^4	$465 \times 10^4/3.5$
F19		ND	465×10^4	$465 \times 10^4/9$
F20		ND	470×10^4	$465 \times 10^4/8$
F21		ND	445×10^4	$465 \times 10^4/9$
F22		ND	470×10^4	$465 \times 10^4/9$
F23		ND	440×10^4	$465 \times 10^4/3.5$
Standard: Tamoxifen		1.75		

intermediate 2, a lower energy state with a ΔE of -9.06 kcal/mol. Intermediates 1 and 2 are potentially in equilibrium, progressing through cyclization to form higher energy intermediate 3 with a ΔE of 8.03 kcal/mol. Finally, intermediate 3 stabilizes upon release of TiO_2 , transitioning to a lower energy intermediate with a ΔE of -5.39 kcal/mol, facilitating the formation of the final product.

The reaction mechanism demonstrates an overall downhill energy profile (Figure 3). Detailed optimized geometries and corresponding ΔE values for intermediates INT1 to INT3 are depicted in Figure 4, providing insights into their structural configurations and energy landscapes.

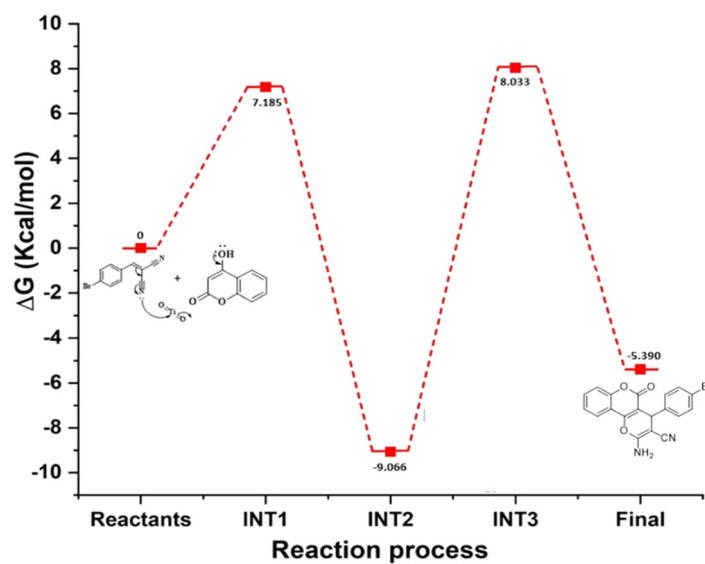


Figure 3. Energy profile diagram illustrating the Gibbs free energy (ΔG) (kcal/mol) for each intermediate in the reaction pathway.

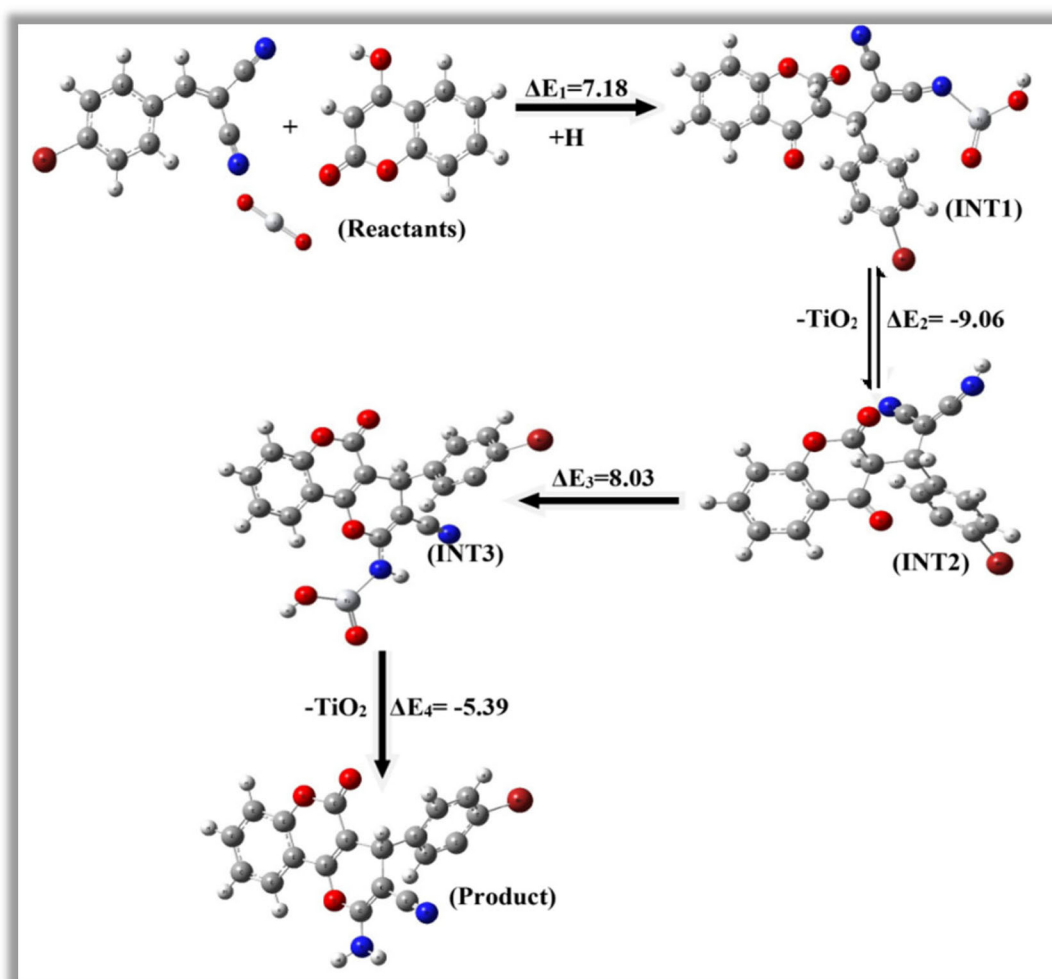


Figure 4. Optimized molecular geometries of intermediate structures along with their corresponding relative energies (ΔE values, kcal/mol).

Efficacy of synthesized compounds on breast cancer cells

The cytotoxic effects of all newly synthesized chromene-3-carbonitriles were assessed on human breast cancer cells (MCF-7) using Alamar Blue assays. IC_{50} values obtained for lead compounds such as **F8**, **F10**, **F12** and **F13** are 0.751, 1.70, 0.68 and 0.52 μ M respectively. Table 1 presents the resulting inhibition concentrations (IC_{50} values), with tamoxifen and doxorubicin used as internal standard drugs and exhibiting IC_{50} values of 1.84 and 2.96 μ M, respectively [Note: For MCF-7 cells viability for these internal standrads, please refer our previous recently published article by Tejaswini et al.⁵⁷] Notably, within the compound series, several methoxy-coumarin derivatives exhibited comparable or even superior anticancer activity relative to their coumarin and fluoro-coumarin counterparts, as evidenced by the data presented in Table 1. These findings highlight the significance of subtle structural modifications on biological activity and describe the potential of specifically substituted chromene-3-carbonitrile derivatives as promising candidates for further anticancer drug development.

Bioinformatic analysis of binding of newly synthesized analogs that target TFF3

The molecular docking method was used to investigate the binding mode including the affinity of the synthesized compounds **F8**, **F10**, **F12** and **F13** with the TFF3. The docking scores calculated for the synthesized compounds were -7.84 , -8.26 , -7.57 , and -7.71 kcal/mole, respectively. The ligand molecule occupied the same interfacial cavity of TFF3 monomers in all docking simulations (Figure 5), resulting in the monomerization of dimeric TFF3.² Docking simulations revealed significant interactions between the ligand-TFF3 complexes. The hydrogen bond interactions between the $-NH_2$ groups of the ligand molecules (**F8**, **F10**, **F12** and **F13**) and the amino acid residues THR58 and GLU54 of TFF3 account for the stability of the ligand-TFF3 complex. In addition to this, the ligand molecules interacted with TFF3 *via* a hydrophobic interaction with the residue VAL19 (Figure 6).

Lead compounds inhibited the in vitro TFF3 activity in mammary carcinoma cells

All compounds were assessed for their ability to inhibit TFF3 expression in MCF-7 cells using Western blot analysis over 48 h. Compounds **F** (1–23) and DMSO were treated under identical experimental conditions, alongside AMPC as a reference drug. The results demonstrated that nearly all tested compounds exhibited superior inhibition of TFF3 compared to the control (DMSO) (Figure 7). This indicates their potential as effective TFF3 inhibitors, highlighting their promise for further development as therapeutic agents against breast cancer and possibly other TFF3-associated malignancies.

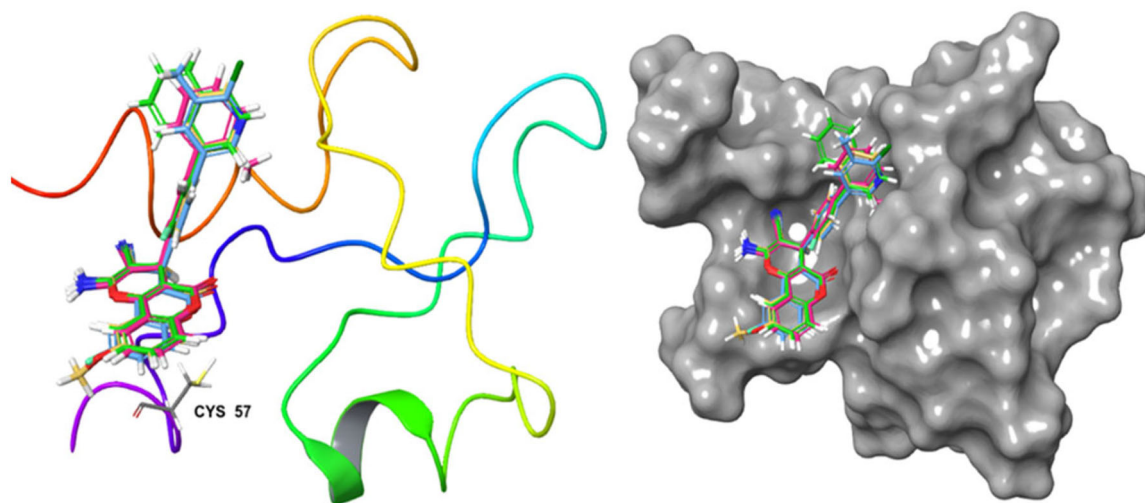


Figure 5. Cartoon representation depicting the surface view and binding poses of compounds **F8** (green), **F10** (magenta), **F12** (yellow), and **F13** (blue) within the interfacial cavity of the TFF3 monomer. These poses provide insights into the molecular interactions and potential binding modes of the compounds with TFF3.

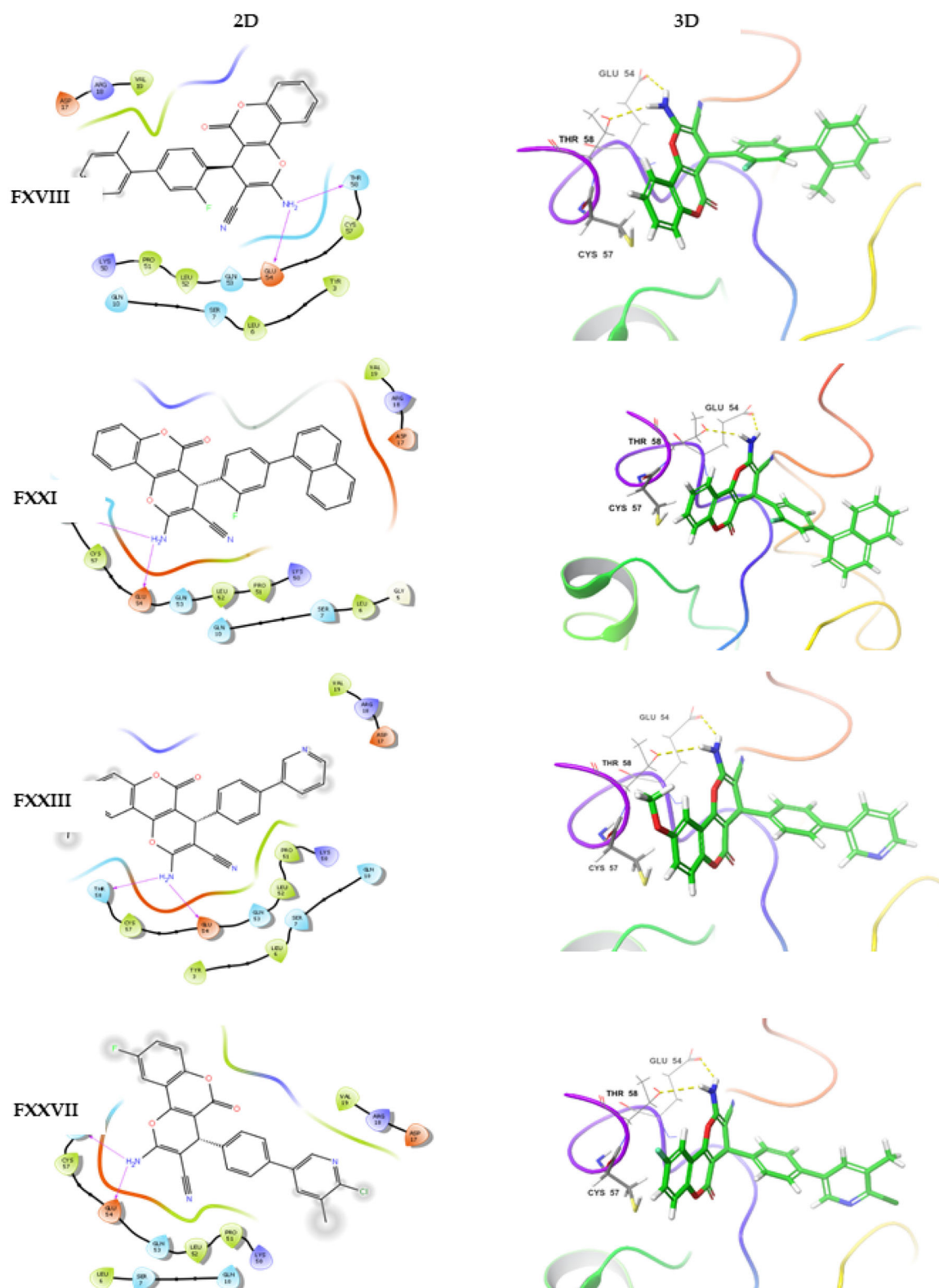


Figure 6. 2D And 3D representations illustrating the molecular interactions of compounds F8, F10, F12 and F13 with the human intestinal TFF3. These representations elucidate the specific binding modes and intermolecular interactions of each compound within the TFF3 protein structure, providing valuable insights into their potential as TFF3 inhibitors or modulators.

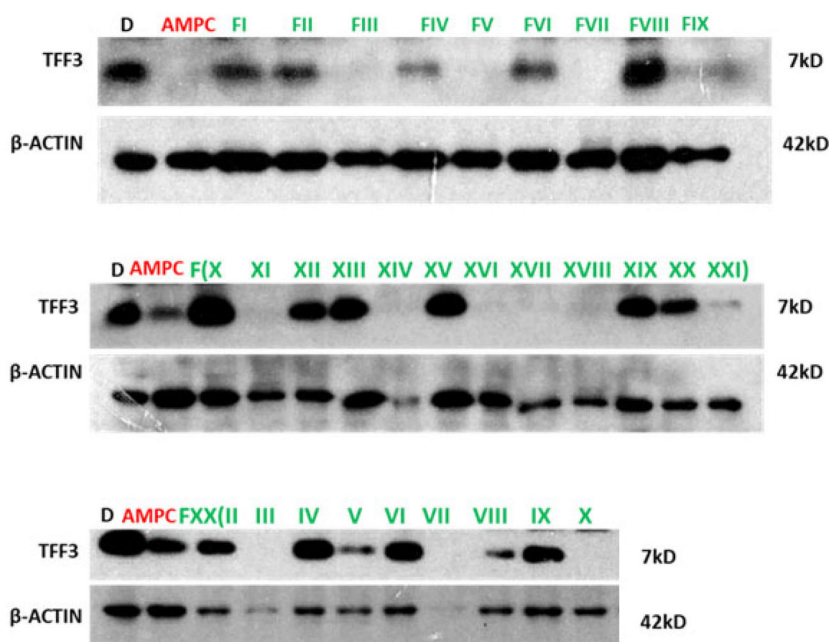


Figure 7. Western Blot analysis depicting the protein expression levels of all synthesized chromene-3-carbonitriles (compounds F1 to F23) on TFF3 in MCF-7 cells following a 48-h treatment period. This assay provides insights into the potential inhibitory effects of these compounds on TFF3 expression, crucial for understanding their therapeutic implications in breast cancer treatment.

Discussion

An efficient and convenient multicomponent cyclization protocol was employed for the synthesis of structurally diverse chromene-based derivatives of synthetic importance. The reaction involves substituted salicylaldehyde, active methylene compounds and electron-rich arenes under optimized catalytic conditions. Similar methodologies have been reported using pTSA-catalyzed reactions in ethanol at 80 °C for constructing chromeno-fused scaffolds.^{58,59} Such multicomponent strategies highlight the synthetic versatility and efficiency of chromene scaffold construction relevant to the present study. Furthermore, nanocatalysis has gained significant attention due to its ability to combine the high activity of homogeneous catalysts with the easy recovery and reusability of heterogeneous systems, offering improved efficiency and selectivity in organic synthesis.⁵⁸ In this regard, a variety of functional nanocatalysts have been developed using simple and scalable synthetic strategies. Silica-decorated ferrite nanoparticles coated with ceric ammonium nitrate (CAN) have shown efficient catalytic performance under solvent-free conditions,⁵⁹ while silica-coated ferrite nanoparticles supported on montmorillonite (K10) and magnetite silica-coated nanoparticle-supported molybdate catalysts have demonstrated structural stability and catalytic versatility in diverse organic transformations.⁶⁰ Montmorillonite (K10)-loaded magnetite silica-coated nanoparticles further highlight the adaptability of nanocatalyst design for sustainable synthesis.⁶¹ Such nanocatalytic platforms have enabled chemoselective reactions, including N-Boc protection of various amines⁶² and green, one-pot reductive amination of functionalized carbonyl compounds.⁶³ These advances collectively support the use of nano-TiO₂ as an efficient and environmentally benign catalyst for the synthesis of biologically active chromene-3-carbonitrile derivatives in the present study.

Furthermore, recent literature clearly demonstrates that nanocatalyst-mediated strategies have become a cornerstone for the efficient and sustainable synthesis of chromene and pyrano-chromene scaffolds. In particular, TiO₂-based nanocatalysts supported on magnetic or hybrid substrates have shown remarkable catalytic activity, enabling one-pot multicomponent reactions with high yields, short reaction times, and excellent catalyst recyclability. For example, TiO₂ nanoparticles supported on Fe₃O₄-coated biochar and nano-cellulose/Ti(IV)/Fe₃O₄ systems have been successfully employed for the synthesis of chromene and related heterocycles under eco-friendly conditions, highlighting the

Industrial Applicability: Pathway From Synthesis to Preclinical Development

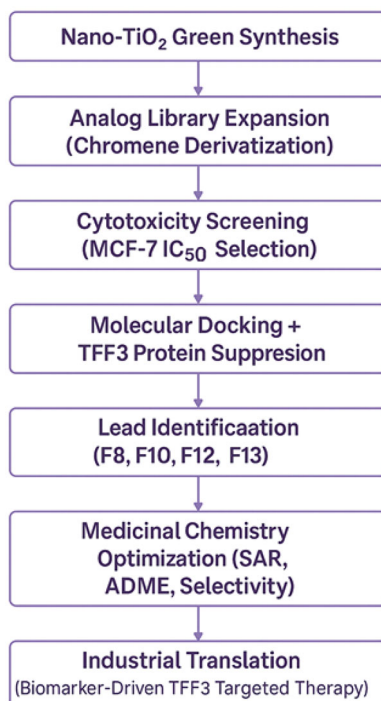


Figure 8. Industrial applicability TFF3-guided therapeutic strategies for ER+ breast cancer patients with high TFF3 expression.

advantages of heterogeneous nanocatalysts in green chemistry applications.^{44–47} More recent studies further confirm that dendritic Fe₃O₄@SiO₂-based nanocatalysts efficiently promote the synthesis of pyrano[3,2-c]chromene-3-carbonitriles with excellent reusability and operational simplicity.^{46,48} Collectively, these studies underscore a clear shift toward nano-engineered catalytic systems for constructing biologically relevant chromene frameworks, reinforcing the contemporary relevance of nanocatalysis in medicinal chemistry.

In this context, the selection of nano-TiO₂ as a catalyst in the present study is strongly supported by both foundational and recent advances in the field. TiO₂ nanomaterials exhibit unique surface acidity, high thermal stability and enhanced mass transfer properties, which are critical for promoting multi-component chromene formation reactions.⁴⁷ Furthermore, emerging photocatalytic TiO₂-based heterojunction systems, such as TiO₂/ZnWO₃ and TiO₂/CuWO₄, have demonstrated superior catalytic performance under mild and sustainable conditions, emphasizing the versatility of TiO₂ nanocomposites in heterocyclic synthesis.^{55,56} Beyond TiO₂, the development of novel metal oxide nanocatalysts, including praseodymium oxide (Pr₆O₁₁), further illustrates the rapid evolution of nanocatalyst design aimed at improving reaction efficiency while adhering to green chemistry principles.⁶⁴ Recent reviews also consolidate these findings by highlighting that magnetic and metal-oxide nanocatalysts consistently enhance reaction rates, selectivity, and recyclability in chromene synthesis, thereby validating nanocatalysis as a mature and impactful strategy in modern synthetic chemistry.⁶⁵ By integrating nano-TiO₂-mediated synthesis with biological validation against TFF3, the present work aligns with and extends these contemporary nanocatalytic approaches toward targeted anticancer drug development.

The results of our study demonstrate that the synthesized chromene-3-carbonitrile derivatives exhibit potent cytotoxicity against MCF-7 breast cancer cells, with lead compounds such as F8, F10, F12, and F13 showing low-micromolar to sub-micromolar IC₅₀ values. These findings are consistent with previous reports that chromene derivatives can exert antitumor effects through diverse mechanisms, including apoptosis induction and kinase modulation.⁴⁹ Molecular docking analyses revealed that the lead

chromene derivatives bind favorably within the TFF3 active site, implying strong interaction potentials with key residues. This supports the hypothesis that these compounds could directly interfere with TFF3's functional domains, which is significant considering TFF3's role in activating pro-survival signaling pathways such as PI3K-AKT and STAT3 in breast carcinoma.⁹ Corroborating the *in silico* results, Western blot analyses showed downregulation of TFF3 protein levels in MCF-7 cells treated with the most active compounds. This suggests that beyond cytotoxic effects, these derivatives may act through targeted suppression of oncogenic TFF3 expression, a strategy aligned with emerging research showing that pharmacologic inhibition of TFF3 enhances the efficacy of standard therapies and reduces cancer stem-like cell behaviors.⁴²

From a synthetic perspective, the use of **nano-TiO₂ catalysis** proved effective for constructing chromene frameworks under thermodynamically favorable conditions, as evidenced by free energy analyses. Nano-TiO₂ catalysts have been widely acknowledged for promoting multicomponent reactions and enabling eco-friendly synthesis with high selectivity and lower environmental impact.⁴⁴ In light of the existing literature, this study extends chromene pharmacology by demonstrating target engagement with a clinically relevant oncogenic protein (TFF3), rather than general cytotoxicity alone. This feature describes the translational potential of these derivatives as targeted anticancer agents that combine scaffold-based cytotoxicity with modulation of a validated cancer target. Future work should explore structure-activity relationships (SAR) to refine interactions with TFF3, evaluate *in vivo* efficacy in xenograft models, and assess effects on downstream signaling pathways (e.g. PI3K-AKT, NF- κ B). Moreover, combining these compounds with established breast cancer therapeutics could reveal synergistic effects, mirroring recent findings where TFF3 inhibitors enhanced sensitivity to endocrine therapies.⁴²

Thus, suppression of TFF3 has been demonstrated to significantly potentiate the therapeutic efficacy of various anti-cancer modalities, including ionizing radiation, gemcitabine, taxanes, and MEK1/2 inhibitors.^{12,17,66} Additionally, targeting TFF3 has shown promise in overcoming resistance to key chemotherapeutic and targeted agents such as 5-FU, anti-estrogen therapies, and HER2-directed treatments.^{12,17,66} These findings elucidate the critical role of TFF3 in mediating drug resistance and suggest that its inhibition could enhance the responsiveness of tumors to conventional and targeted therapies. High-throughput screening of anti-cancer compounds has provided further insights into the therapeutic potential of TFF3 inhibition. Specifically, the small-molecule inhibitor AMPC was found to act synergistically with drugs targeting four distinct RTKs including EGFR, VEGF-Receptor, c-KIT, and c-MET in MDA-MB-361 and BT474 breast cancer cell lines. These RTKs are well-established drivers of oncogenic signaling and have been implicated in tumor progression, angiogenesis, and metastasis.⁶⁷ Concurrent targeting of these pathways alongside TFF3 inhibition presents a promising strategy to disrupt compensatory survival mechanisms within cancer cells. Indeed, therapeutic approaches directed against EGFR, VEGFR, and c-MET, whether as monotherapies or in combination regimens, have exhibited substantial anti-tumor effects by impairing cellular proliferation, invasion, and metastatic dissemination.^{68,69} However, acquired resistance remains a significant challenge in RTK-targeted therapy, necessitating novel combinatorial strategies to sustain treatment efficacy. Mechanistically, TFF3 has been implicated in the modulation of EGFR activity through its competitive interaction with LINGO2. Previous reports described that TFF3 disrupts EGFR-LINGO2 complexes, thereby facilitating sustained activation of EGFR signaling.⁷⁰ Consistently, pharmacological inhibition of TFF3 using AMPC can attenuate EGFR activity in ER+ breast cancer cells.³⁹ Given the central role of EGFR in tumor progression, these findings highlight TFF3 as a key regulator of RTK-mediated oncogenic signaling. Beyond EGFR, TFF3 has also been shown to modulate multiple intracellular signaling cascades, including the p44/42-MAPK pathway,¹² PI3K/AKT pathway,^{20,71} and STAT3 signaling.^{10,72} These signaling axes are integral to cellular proliferation, survival, and chemoresistance. The ability of TFF3 to influence such diverse signaling pathways suggests its broader involvement in RTK-mediated oncogenic processes, positioning it as a compelling therapeutic target. Collectively, the current body of evidence, including the findings presented in this study, supports the notion that TFF3 depletion or inhibition can enhance the therapeutic efficacy of RTK inhibitors in cancer treatment. By mitigating the oncogenic crosstalk mediated by RTKs, TFF3-targeted strategies may serve as an effective means to counteract drug resistance and improve clinical outcomes in cancers driven by aberrant RTK signaling.^{39,66} Future

investigations should explore the translational potential of combining TFF3 inhibitors with established RTK-targeted therapies to optimize anti-cancer treatment regimens and overcome resistance mechanisms.⁶⁷ Our previous report described that targeting TFF3 in conjunction with c-MET inhibition presents a compelling therapeutic approach for the effective management of ER+/HER2+ MC, potentially enhancing treatment efficacy and overcoming resistance mechanisms.⁶⁷ The findings of this study provide compelling evidence supporting the potential of chromene-3-carbonitrile derivatives as novel therapeutic agents for breast adenocarcinomas. The significant cytotoxicity observed in breast cancer cells indicates the efficacy of these compounds in inhibiting cancer cell proliferation. The molecular docking studies revealed strong binding interactions between TFF3 and lead compound F18 suggesting their capability to interfere with above mentioned TFF3-mediated oncogenic signaling pathways.

IC₅₀ values obtained for these lead compounds demonstrate their variable potency, with F18 exhibiting the highest efficacy (3.41 μ M). These results align with previous studies that emphasize the role of TFF3 as a key regulator of tumor progression, highlighting the necessity of targeted therapeutic strategies. Furthermore, the thermodynamic feasibility of the synthetic pathway, as indicated by energy profile analysis, describes the stability and efficiency of the newly synthesized compounds, thereby enhancing their potential for further development.

Western blot analysis confirmed the inhibitory effects of these derivatives on TFF3 expression, providing mechanistic insights into their mode of action. The downregulation of TFF3 protein levels suggests that these compounds may suppress TFF3-mediated signaling cascades, thereby exerting their anticancer effects. This finding is particularly significant given the role of TFF3 in promoting tumor growth, survival, and metastasis. Collectively, these results support the therapeutic potential of chromene-3-carbonitrile derivatives in targeting TFF3 for breast cancer treatment.

Significance

This work advances knowledge in three ways. First, conceptually, we demonstrate that chromene-3-carbonitrile derivatives can act as small-molecule modulators of TFF3 in breast cancer cells, linking a well-studied heterocyclic scaffold to a specific oncogenic protein target. Second, methodologically, we integrate nano-TiO₂ catalysis, thermodynamic feasibility analysis, molecular docking and biochemical validation as a streamlined discovery pipeline showing that green nanocatalysis can be combined with target-oriented medicinal chemistry. Third, translationally, we provide lead compounds with sub- to low-micromolar potency and evidence of TFF3 downregulation, which together represent promising starting points for further medicinal chemistry and preclinical development aimed at overcoming endocrine resistance. These contributions fill the gap between TFF3 biology (where inhibition has shown therapeutic benefit in preclinical models)^{39,42,50} and practical small-molecule scaffold development for TFF3 targeting. **How this differs from prior work:** Previous reports have demonstrated (a) the biological importance and therapeutic promise of TFF3 inhibition and (b) anticancer activity of various chromene series toward breast cancer lines (see Abdelall;⁵¹ Alneyadi⁵²) Our study uniquely connects these areas by producing chromene-3-carbonitrile leads specifically shown to bind and reduce TFF3 levels in MCF-7 cells, thereby enabling hypothesis-driven follow-up (SAR, PK/PD, combination therapy) that was not available previously.

Industrial applicability

The present findings carry several implications for industrial and translational applications. (1) **Scalable, green synthesis:** the nano-TiO₂ catalyzed protocol we used to be compatible with multicomponent reactions, operates under mild conditions and can be adapted to continuous-flow or larger batch processing features attractive to process chemists seeking cost-effective and environmentally friendlier synthesis of heterocyclic libraries. Recent advances demonstrate that TiO₂-based photocatalytic/heterojunction systems can be scaled and tuned for higher throughput synthesis of chromene cores. (2) **Lead-generation for medicinal chemistry:** the sub-micromolar lead compounds (F8/F10/F12/F13) provide drug discovery starting points for optimization of potency, selectivity and ADME properties; scaffold-hopping and parallel medicinal chemistry can produce analogues amenable to

Table 2. Industrial applicability: pathway from synthesis to preclinical development.

Stage	Industrial relevance	Key outputs / advantages	Application to this study
1. Green synthesis using Nano-TiO ₂	- Scalable nanocatalyst-based process compatible with batch and continuous flow. - Low-cost, recyclable catalyst supports green-chemistry metrics (reduced solvent load, mild conditions).	- High product yields - Reduced energy and waste - Catalyst reusability reduces cost of goods (COGs)	Chromene-3-carbonitrile derivatives synthesized <i>via</i> nano-TiO₂ provide a scalable, environmentally friendly production route for early drug candidates.
2. Library Expansion & High-throughput derivatization	- Pharmaceutical R&D requires rapid generation of analog libraries. - Chromene scaffolds are amenable to multicomponent reactions and parallel synthesis.	- Structural diversity - Fast generation of analog series for SAR studies	Current synthetic method can be extended to produce larger chromene libraries optimized for potency, solubility, and TFF3-binding.
3. Lead identification (Potency screening)	Industry standard IC₅₀ threshold for early leads is usually low-micromolar or sub-micromolar.	Potent hits selected for downstream optimization	F8, F10, F12, F13 exhibit sub-micromolar IC₅₀ values, meeting industry criteria for advancement and justifying investment in SAR optimization.
4. Mechanism-based target validation	- Target validation before investment in medicinal chemistry reduces failure rates. - Mechanistic linkage increases patentability and regulatory strength.	- Confirmation of target engagement - Helps define MoA	Strong molecular docking scores and TFF3 downregulation on Western blot confirm target engagement, supporting industrial confidence.
5. Lead optimization (SAR, DMPK, Selectivity)	- Medicinal chemistry: optimize potency, lipophilicity, stability, solubility, off-target activity. - ADME profiling and toxicity screening.	- Improved drug-like properties - Reduction of toxicity liabilities (CYP, hERG, genotoxicity)	Chromene scaffold is chemically tractable; optimized analogs can improve selectivity against TFF3 and enhance pharmacokinetics.
6. Preclinical pharmacology	Testing in 2D/3D cancer models, patient-derived organoids, and <i>in vivo</i> xenografts.	Efficacy, tolerability, biodistribution, PK/PD	TFF3 has a role in endocrine resistance; optimized compounds could demonstrate synergy with tamoxifen or aromatase inhibitors in preclinical models.
7. Early Toxicology & Safety profiling	Mandatory stage before IND filing.	- Identification of safety margins - Dose-limiting toxicities	Chromene derivatives previously showed acceptable safety in early <i>in vitro</i> screens; optimization may further improve safety windows.
8. Translational Potential & Companion diagnostics	TFF3 is a measurable biomarker in tissues and fluids, enabling patient selection strategies.	- Supports personalized medicine - Enhances clinical trial success	Potential development of TFF3-guided therapeutic strategies for ER+ breast cancer patients with high TFF3 expression.

preclinical development. (3) **Translational value & combinability:** because TFF3 inhibition has been shown to reverse endocrine resistance and potentiate chemotherapies in preclinical models, industrial development of TFF3-directed small molecules could be combined with existing endocrine or targeted therapies to address resistant ER+ breast cancers. (4) **Biomarker-guided development:** TFF3 is measurable in tumor tissue and circulation in some settings, enabling biomarker-driven patient selection strategies in early clinical development (Figure 8).

Practical next steps for industry translation

(1) Undertake process optimization of the nano-TiO₂ synthesis route (yield, catalyst recovery, solvent minimization). (2) Conduct medicinal chemistry SAR to improve metabolic stability and selectivity (CYP and off-target screens) (Table 2). (3) Test lead compounds in *in vivo* efficacy and safety models and investigate combination regimens with endocrine agents (e.g. tamoxifen or aromatase inhibitors) given preclinical synergy reported for TFF3 blockade.

Materials and methods

The progress of reactions was continuously monitored using thin-layer chromatography (TLC), a versatile analytical technique. For TLC analysis, Merck silica gel 60 F254 plates were utilized as the

stationary phase, while a mixture of ethyl acetate and hexane served as the mobile phase. Visualization of separated compounds was achieved under UV light, facilitating the detection and characterization of reaction intermediates and products.

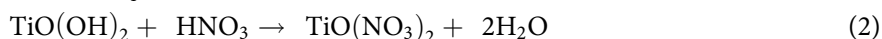
Proton nuclear magnetic resonance (^1H NMR) spectra were obtained predominantly by an Agilent instrument, complemented by occasional recordings on a Bruker Avance-400 and JEOL NMR spectrometer, all employing DMSO- d_6 as the solvent. Carbon-13 nuclear magnetic resonance (^{13}C NMR) spectra were predominantly collected on an Agilent NMR instrument, with **supplementary files** data obtained on a Bruker Avance-400 and JEOL instrument, using solvents such as CDCl_3 and DMSO- d_6 . Chemical shifts were referenced relative to tetramethylsilane (TMS), facilitating the accurate assignment of molecular structures.

Mass spectrometry analyses were conducted on an Agilent LC-MS system, providing additional insights into the molecular weights and fragmentation patterns of synthesized compounds.

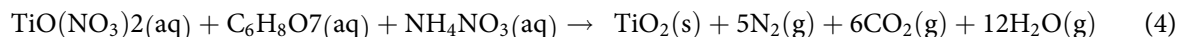
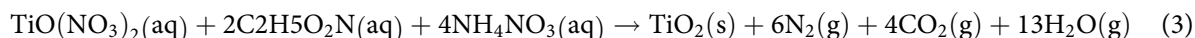
Throughout the study, commercially available reagent-grade solvents and chemicals were employed without additional purification steps. For purification purposes, column chromatography was performed using silica gel (60–120 mesh), enabling the isolation and separation of target compounds based on their polarity and molecular size.

Synthesis of nano TiO_2

Nano TiO_2 was synthesized by combustion synthesis using titanyl nitrate ($\text{TiO}(\text{NO}_3)_2$) and fuels like glycine ($\text{C}_2\text{H}_5\text{O}_2\text{N}$) or citric acid ($\text{C}_6\text{H}_8\text{O}_7$) based on the total oxidizing and reducing valency of the oxidizer and the fuel, which served as a numerical coefficient for the stoichiometric balance so that the equivalence ratio was equal to unity (i.e. total oxidizing valency/total reducing valency (O/F) = 1) and the energy released was maximum. Ammonium nitrate (NH_4NO_3) was sometimes added for the complete balance of the total oxidizing and reducing valency of the oxidizer and the fuel. According to concepts used in propellant chemistry, the elements, C, H, and Ti^{4+} have valencies of +4, +1, and +4, respectively. The element oxygen was considered an oxidizing element with a valency of -2 . The valency of nitrogen was considered to be zero. $\text{TiO}(\text{NO}_3)_2$ was produced *in situ* by the hydrolysis of $\text{Ti}(\text{i-OPr})_4$ followed by treatment with HNO_3 .⁷³



The combustion reactions are represented by the following balanced equations:



General procedure for the synthesis of 2-amino chromene-3-carbonitriles derivatives

A one-pot, three-component condensation reaction involving substituted coumarin (**A**) (1 mmol) was reacted with different aryl aldehydes (**B**) (1 mmol) and malononitrile (**C**) (3 mmol), catalyzed by nano- TiO_2 (0.01 mmol) under reflux in ethanol for 3–12 h to afford 2-amino-4-aryl-4H-benzo[h]chromene-3-carbonitrile derivatives (**D**). After completion of the reaction, as confirmed by TLC, the solvent was removed under reduced pressure. The resulting crude mixture was extracted with ethyl acetate ($3 \times 20\text{ml}$), and the combined organic layers were concentrated under vacuum. The residue was subsequently purified by column chromatography to afford pure compound **D**.

General procedure for synthesis of the 2-amino-5-oxo-4-(4-(aryl)phenyl)-4,5dihydropyrano-[3,2-c]chromene-3-carbonitrile analogues

The synthesized compound (**D**) (1 mmol) were reacted with various boronic acids (**E**) (1.2 mmol) under Suzuki coupling conditions. The reaction was carried out at 70°C in the presence of

$\text{Pd(dppf)}_2\text{Cl}_2$ (**0.001 mmol**) as the catalyst and potassium carbonate (**0.5 mmol**) as the base, using a mixture of water (1 mL) and tetrahydrofuran (4 mL) as the solvent system. After 8–10 h, reaction progress was monitored by TLC (hexane:ethyl acetate, 8:2). Upon completion, the crude mixture was subjected to column chromatography using hexane–ethyl acetate mixture to yield pure compound (**F 1–23**).

Characterization data

2-amino-5-oxo-4-(4'-(trifluoromethyl)-[1,1'-biphenyl]-4-yl)-4,5-dihydropyrano[3,2-c]chromene-3-carbonitrile, F1

Off White solid; mp 258–260 °C; 89% yield; IR ν_{max} : 3300 cm^{-1} $\nu_{(\text{NH}_2)}$, 2100 cm^{-1} $\nu_{(\text{CN})}$, 1710 cm^{-1} $\nu_{(\text{C}=\text{O})}$; ^1H NMR (DMSO- d_6 , 400 MHz) δ : 7.91 (d, $J=8.5$ Hz, 1H), 7.8–7.85 (m, 2H) 7.80–7.78 (m, 2H), 7.68–7.67 (m, 3H), 7.52–7.49 (m, 4H), 7.39 (d, $J=7.5$ Hz, 2H), 4.50 (s, 1H); ^{13}C NMR (DMSO- d_6 , 100 MHz) δ : 159.6, 158.0, 153.6, 152.2, 143.8, 143.7, 137.5, 133.0, 128.5, 127.6, 127.4, 127.3, 125.8, 124.7, 122.5, 119.3, 116.6, 112.9, 103.8, 57.6, 39.2; LCMS (ESI): m/z Calcd for $\text{C}_{26}\text{H}_{15}\text{F}_3\text{N}_2\text{O}_3$ $[\text{M} + \text{H}]^+$: 461.1113; Found: 461.1119.

2-amino-4-(4-(6-methylpyridin-3-yl)phenyl)-5-oxo-4,5-dihydropyrano[3,2-c]chromene-3-carbonitrile, F2

White solid; mp 252–254 °C; 90% yield; IR ν_{max} : 3300 cm^{-1} $\nu_{(\text{NH}_2)}$, 2100 cm^{-1} $\nu_{(\text{CN})}$, 1728 cm^{-1} $\nu_{(\text{C}=\text{O})}$; ^1H NMR (DMSO- d_6 , 400 MHz) δ : 8.72 (d, $J=2.5$ Hz, 1H), 7.94–7.89 (m, 2H), 7.64–7.62 (m, 1H), 7.49–7.47 (m, 2H), 7.43–7.37 (m, 4H), 7.36 (d, $J=8$ Hz, 2H), 7.32 (d, $J=8$ Hz, 1H), 4.51 (s, 1H), 2.49 (s, 3H); ^{13}C NMR (DMSO- d_6 , 100 MHz) δ : 159.6, 158.0, 156.8, 153.5, 152.2, 146.7, 142.9, 134.4, 132.9, 132.41, 128.4, 126.9, 124.7, 123.2, 122.5, 116.6, 103.8, 57.7, 39.0, 23.6; LCMS (ESI): m/z Calcd for $\text{C}_{25}\text{H}_{17}\text{N}_3\text{O}_3$ $[\text{M} + \text{H}]^+$: 408.1348; Found: 408.7561.

2-amino-4-(4'-chloro-3-fluoro-[1,1'-biphenyl]-4-yl)-5-oxo-4,5-dihydropyrano[3,2-c]chromene-3-carbonitrile, F3

Off White solid; mp 220–222 °C; 88% yield; IR ν_{max} : 3300 cm^{-1} $\nu_{(\text{NH}_2)}$, 2100 cm^{-1} $\nu_{(\text{CN})}$, 1701 cm^{-1} $\nu_{(\text{C}=\text{O})}$; ^1H NMR (DMSO- d_6 , 400 MHz) δ : 7.90–7.87 (m, 1H), 7.69–7.67 (m, 3H), 7.53–7.36 (m, 7H), 7.14 (s, 1H), 6.65 (s, 1H) 4.73 (s, 1H); LCMS (ESI): m/z Calcd for $\text{C}_{25}\text{H}_{14}\text{ClFN}_2\text{O}_3$ $[\text{M} + \text{H}]^+$: 445.0755; Found: 445.1756.

2-amino-4-(2-fluoro-4-(pyridin-4-yl)phenyl)-5-oxo-4,5-dihydropyrano[3,2-c]chromene-3-carbonitrile, F4

Off White solid; mp 228–230 °C; 93% yield; IR ν_{max} : 3300 cm^{-1} $\nu_{(\text{NH}_2)}$, 2100 cm^{-1} $\nu_{(\text{CN})}$, 1727 cm^{-1} $\nu_{(\text{C}=\text{O})}$; ^1H NMR (DMSO- d_6 , 400 MHz) δ : 8.62–8.61 (m, 2H), 7.88 (d, $J=8$ Hz, 1H) 7.72–7.56 (m, 5H), 7.502–7.439 (m, 5H), 4.77 (s, 1H); ^{13}C NMR (DMSO- d_6 , 100 MHz) δ : 162.3, 159.9, 159.5, 158.8, 154.4, 152.6, 150.7, 145.8, 138.7, 133.6, 131.6, 131.3, 131.2, 123.2, 122.9, 121.7, 119.5, 117.1, 114.6, 114.3, 113.3, 102.8, 56.4, 31.6; LCMS (ESI): m/z Calcd for $\text{C}_{24}\text{H}_{14}\text{FN}_3\text{O}_3$ $[\text{M} + \text{H}]^+$: 412.1097; Found: 411.9979.

2-amino-4-(4-(6-chloro-5-methylpyridin-3-yl)-2-fluorophenyl)-5-oxo-4,5-dihydropyrano[3,2-c]chromene-3-carbonitrile, F5

Off White solid; mp 246–248 °C; 88% yield; IR ν_{max} : 3300 cm^{-1} $\nu_{(\text{NH}_2)}$, 2100 cm^{-1} $\nu_{(\text{CN})}$, 1702 cm^{-1} $\nu_{(\text{C}=\text{O})}$; ^1H NMR (DMSO- d_6 , 400 MHz) δ : 8.56–8.55 (m, 1H), 8.13–8.12 (m, 1H), 7.89–7.88 (m, 1H), 7.73–7.68 (m, 1H), 7.58–7.55 (m, 1H), 7.51–7.39 (m, 6H), 4.75 (s, 1H), 2.356 (s, 3H); LCMS (ESI): m/z Calcd for $\text{C}_{25}\text{H}_{15}\text{ClFN}_3\text{O}_3$ $[\text{M} + \text{H}]^+$: 460.0864; Found: 460.0386.

2-amino-4-(3-fluoro-4'-(trifluoromethyl)-[1,1'-biphenyl]-4-yl)-5-oxo-4,5-dihydropyrano[3,2-c]chromene-3-carbonitrile, F6

Off White solid; mp 260–262 °C; 84% yield; IR ν_{max} : 3300 cm^{-1} $\nu_{(\text{NH}_2)}$, 2100 cm^{-1} $\nu_{(\text{CN})}$, 1698 cm^{-1} $\nu_{(\text{C}=\text{O})}$; ^1H NMR (DMSO- d_6 , 400 MHz) δ : 8.07–8.05 (m, 1H), 7.95–7.87 (m, 3H), 7.78–7.70 (m, 2H),

7.56–7.41 (m, 6H), 4.75 (s, 1H); LCMS (ESI): m/z Calcd for $C_{26}H_{14}F_4N_2O_3$ $[M + H]^+$: 479.1018; Found: 479.1022.

2-amino-4-(2-fluoro-4-(6-methylpyridin-3-yl)phenyl)-5-oxo-4,5-dihydropyrano[3, 2-c]chromene-3-carbonitrile, F7

Off White solid; mp 228–230 °C; 86% yield; ^1H NMR (DMSO- d_6 , 400 MHz) δ : 8.78 (s, 1H), 8.77–8.05 (m, 1H), 7.89 (d, J = 8Hz, 1H), 7.72–7.68 (m, 1H), 7.56–7.48 (m, 1H), 7.47–7.36 (m, 7H), 4.74 (s, 1H), 2.47 (s, 3H); ^{13}C NMR (DMSO- d_6 , 100 MHz) δ : 162.3, 159.9, 159.9, 158.7, 157.4, 154.4, 152.6, 146.5, 135.8, 133.5, 131.9, 131.4, 129.9, 129.8, 125.2, 124.1, 123.3, 122.9, 119.5, 117.1, 113.3, 102.9, 56.5, 31.6, 23.6; LCMS (ESI): m/z Calcd for $C_{25}H_{16}FN_3O_3$ $[M + H]^+$: 426.1253; Found: 426.0078.

2-amino-4-(3-fluoro-2'-methyl-[1,1'-biphenyl]-4-yl)-5-oxo-4,5-dihydropyrano[3,2-c]chromene-3-carbonitrile, F8

Yellow solid; mp 258–260 °C; 86% yield; ^1H NMR (DMSO- d_6 , 400 MHz) δ : 7.65–7.62 (m, 1H), 7.60–7.55 (m, 2H), 7.53–7.51 (m, 1H), 7.44–7.38 (m, 2H), 7.30–7.13 (m, 7H), 4.49 (s, 1H), 2.18 (s, 3H); LCMS (ESI): m/z Calcd for $C_{26}H_{17}FN_2O_3$ $[M + H]^+$: 425.1301; Found: 425.1323.

2-amino-4-(3-fluoro-3'-methoxy-[1,1'-biphenyl]-4-yl)-5-oxo-4,5-dihydropyrano[3, 2-c]chromene-3-carbonitrile, F9

Yellow solid; mp 252–254 °C; 82% yield; IR ν_{max} : 3300 cm^{-1} $\nu_{(NH_2)}$, 2100 cm^{-1} $\nu_{(CN)}$, 1710 cm^{-1} $\nu_{(C=O)}$; ^1H NMR (DMSO- d_6 , 400 MHz) δ : 7.89 (d, J = 8Hz, 1H), 7.70–7.68 (m, 1H), 7.50–7.49 (m, 6H), 7.36–7.31 (m, 2H), 7.21–7.17 (m, 2H), 6.93–6.90 (m, 1H), 3.78 (s, 3H), 4.73 (s, 1H); ^{13}C NMR (DMSO- d_6 , 100 MHz) δ : 162.2, 160.2, 159.7, 158.8, 154.4, 152.6, 141.8, 140.5, 133.5, 131.1, 130.5, 129.4, 125.2, 123.4, 122.9, 119.8, 119.5, 117.1, 114.4, 114.1, 113.3, 112.7, 103.1, 56.7, 55.6, 31.6; LCMS (ESI): m/z Calcd for $C_{26}H_{17}FN_2O_4$ $[M + H]^+$: 441.1250; Found: 441.1263.

2-amino-4-(2-fluoro-4-(naphthalen-1-yl)phenyl)-5-oxo-4,5-dihydropyrano[3,2-c]chromene-3-carbonitrile, F10

Off white solid; mp 250–252 °C; 90% yield; IR ν_{max} : 3300 cm^{-1} $\nu_{(NH_2)}$, 2100 cm^{-1} $\nu_{(CN)}$, 1703 cm^{-1} $\nu_{(C=O)}$; ^1H NMR (DMSO- d_6 , 400 MHz) δ : 7.98–7.76 (m, 3H), 7.76–7.69 (m, 2H), 7.55–7.41 (m, 9H), 7.29–7.23 (m, 2H) 4.80 (s, 1H); LCMS (ESI): m/z Calcd for $C_{29}H_{17}FN_2O_3$ $[M + H]^+$: 461.1301; Found: 461.1331.

2-amino-9-fluoro-5-oxo-4-(4-(pyridin-3-yl)phenyl)-4,5-dihydropyrano[3,2-c]chromene-3-carbonitrile, F11

Off white solid; mp 258–260 °C; 90% yield; ^1H NMR (DMSO- d_6 , 400 MHz) δ : 8.85 (s, 1H), 8.53 (d, J = 4Hz, 1H), 8.04 (d, J = 8Hz, 1H), 7.66–7.60 (m, 3H), 7.58–7.55 (m, 1H), 7.53–7.51 (m, 1H), 7.49–7.45 (m, 1H), 7.44–7.42 (m, 2H), 7.38–7.36 (m, 2H), 4.51 (s, 1H); ^{13}C NMR (DMSO- d_6 , 100 MHz) δ : 159.8, 158.4, 153.3, 149.0, 147.8, 143.5, 136.4, 135.8, 134.8, 128.9, 127.6, 124.4, 120.8, 120.6, 119.5, 119.3, 119.3, 108.8, 108.8, 105.0, 58.2, 37.2; LCMS (ESI): m/z Calcd for $C_{24}H_{14}FN_3O_3$ $[M + H]^+$: 412.1097; Found: 412.8026.

2-amino-8-methoxy-5-oxo-4-(4-(pyridin-3-yl)phenyl)-4,5-dihydropyrano[3,2-c]chromene-3-carbonitrile, F12

Off white solid; mp 232–234 °C; 91% yield; IR ν_{max} : 3300 cm^{-1} $\nu_{(NH_2)}$, 2100 cm^{-1} $\nu_{(CN)}$, 1724 cm^{-1} $\nu_{(C=O)}$; ^1H NMR (DMSO- d_6 , 400 MHz) δ : 8.83 (s, 1H), 8.52–8.57 (m, 1H), 8.02–8.00 (m, 1H), 7.77 (d, J = 8Hz, 1H), 7.65–7.45 (m, 2H), 7.49–7.43 (m, 1H), 7.39–7.29 (m, 4H) 7.10–7.05 (m, 2H), 4.45 (s, 1H), 3.84 (s, 3H); LCMS (ESI): m/z Calcd for $C_{25}H_{17}N_3O_4$ $[M + H]^+$: 424.1297; Found: 424.1625.

2-amino-4-(4-(6-chloro-5-methylpyridin-3-yl)phenyl)-9-fluoro-5-oxo-4,5-dihydro pyrano [3,2-c]chromene-3-carbonitrile, F13

Off white solid; mp 244–246 °C; 84% yield; IR ν_{max} : 3300 cm^{-1} $\nu_{(NH_2)}$, 2100 cm^{-1} $\nu_{(CN)}$, 1710 cm^{-1} $\nu_{(C=O)}$; ^1H NMR (DMSO- d_6 , 400 MHz) δ : 8.49 (s, 1H), 8.04 (s, 1H), 7.64–7.50 (m, 5H), 7.37–7.35 (m,

2H), 7.40 (s, 2H), 4.49 (s, 1H), 2.34 (s, 3H); LCMS (ESI): m/z Calcd for $C_{25}H_{15}ClFN_3O_3$ $[M + H]^+$: 460.0864; Found: 460.8163.

2-amino-4-(4-(2-fluoro-3-methylpyridin-4-yl)phenyl)-8-methoxy-5-oxo-4,5-dihydropyrano[3,2-c]chromene-3-carbonitrile, F14

Off white solid; mp 220–222 °C; 81% yield; IR $\nu_{\max}$: 3300 cm^{-1} $\nu_{(\text{NH}_2)}$, 2100 cm^{-1} $\nu_{(\text{CN})}$, 1723 cm^{-1} $\nu_{(\text{C}=\text{O})}$; ^1H NMR (DMSO- d_6 , 400 MHz) δ : 8.1–8.2 (m, 1H), 7.8–7.9 (m, 1H), 7.5–7.3 (m, 6H), 7.3–7 (m, 3H), 4.5 (s, 1H), 3.9 (s, 3H), 2.2 (s, 3H); LCMS (ESI): m/z Calcd for $C_{26}H_{18}FN_3O_4$ $[M + H]^+$: 456.1359; Found: 456.1363.

2-amino-8-methoxy-5-oxo-4-(4'-(trifluoromethyl)-[1,1'-biphenyl]-4-yl)-4,5-dihydropyrano[3,2-c]chromene-3-carbonitrile, F15

Off white solid; mp 180–182 °C; 91% yield; IR $\nu_{\max}$: 3300 cm^{-1} $\nu_{(\text{NH}_2)}$, 2100 cm^{-1} $\nu_{(\text{CN})}$, 1711 cm^{-1} $\nu_{(\text{C}=\text{O})}$; ^1H NMR (DMSO- d_6 , 400 MHz) δ : 7.84–7.82 (m, 2H), 7.78–7.75 (m, 3H), 7.65–7.63 (m, 2H), 7.39 (s, 1H), 7.35–7.33 (m, 2H), 7.08–7.05 (m, 1H), 7.02–7.03 (m, 2H), 4.45 (s, 1H), 3.832 (s, 3H); LCMS (ESI): m/z Calcd for $C_{27}H_{17}F_3N_2O_4$ $[M + H]^+$: 491.1218; Found: 491.1228.

2-amino-8-methoxy-4-(4-(6-methylpyridin-3-yl)phenyl)-5-oxo-4,5-dihydropyrano[3,2-c]chromene-3-carbonitrile, F16

Off white solid; mp 214–216 °C; 82% yield; IR $\nu_{\max}$: 3300 cm^{-1} $\nu_{(\text{NH}_2)}$, 2100 cm^{-1} $\nu_{(\text{CN})}$, 1732 cm^{-1} $\nu_{(\text{C}=\text{O})}$; ^1H NMR (DMSO- d_6 , 400 MHz) δ : 8.69 (d, $J = 1.6\text{ Hz}$, 1H), 7.89 (dd, $J = 2.4\text{ Hz}$, 1H), 7.77–7.75 (m, 1H), 7.61–7.59 (m, 2H), 7.33–7.23 (m, 3H), 7.38 (s, 2H), 7.079–7.052 (m, 1H), 4.43 (s, 1H), 3.83 (s, 3H), 2.46 (s, 3H); LCMS (ESI): m/z Calcd for $C_{26}H_{19}N_3O_4$ $[M + H]^+$: 438.1453; Found: 438.1468.

2-amino-9-fluoro-4-(4-(naphthalen-1-yl)phenyl)-5-oxo-4,5-dihydropyrano[3,2-c]chromene-3-carbonitrile, F17

White solid; mp 250–252 °C; 91% yield; IR $\nu_{\max}$: 3300 cm^{-1} $\nu_{(\text{NH}_2)}$, 2100 cm^{-1} $\nu_{(\text{CN})}$, 1705 cm^{-1} $\nu_{(\text{C}=\text{O})}$; ^1H NMR (DMSO- d_6 , 400 MHz) δ : 7.96–7.89 (m, 2H), 7.77–7.71 (m, 2H), 7.59–7.40 (m, 12H), 4.54 (s, 1H); ^{13}C NMR (DMSO- d_6 , 100 MHz) δ : 159.9, 158.6, 153.4, 149.0, 142.7, 139.2, 133.9, 131.2, 130.4, 128.8, 128.1, 127.3, 126.8, 126.3, 125.9, 123.6, 120.9, 120.6, 119.6, 119.2, 114.7, 114.6, 109.0, 108.8, 105.3, 58.1, 37.2; LCMS (ESI): m/z Calcd for $C_{29}H_{17}FN_2O_3$ $[M + H]^+$: 461.1301; Found: 461.1321.

2-amino-4-(4-(6-chloro-5-methylpyridin-3-yl)-2-fluorophenyl)-9-fluoro-5-oxo-4,5-dihydropyrano[3,2-c]chromene-3-carbonitrile, F18

Off White solid; mp 244–246 °C; 93% yield; IR $\nu_{\max}$: 3300 cm^{-1} $\nu_{(\text{NH}_2)}$, 2100 cm^{-1} $\nu_{(\text{CN})}$, 1729 cm^{-1} $\nu_{(\text{C}=\text{O})}$; ^1H NMR (DMSO- d_6 , 400 MHz) δ : 8.56–8.55 (m, 1H), 8.13–8.12 (m, 1H), 7.65–7.49 (m, 5H), 7.44–7.40 (m, 3H), 4.760 (s, 1H), 2.35 (s, 3H); ^{13}C NMR (DMSO- d_6 , 100 MHz) δ : 162.3, 159.9, 158.6, 157.5, 153.8, 150.4, 149.1, 145.5, 138.6, 137.5, 133.9, 132.6, 131.5, 123.5, 119.4, 119.3, 114.6, 114.5, 114.4, 108.8, 108.5, 103.7, 56.5, 31.6, 19.5; LCMS (ESI): m/z Calcd for $C_{25}H_{14}ClF_2N_3O_3$ $[M + H]^+$: 478.0770; Found: 478.0770.

2-amino-4-(4-(6-chloro-5-methylpyridin-3-yl)-2-fluorophenyl)-8-methoxy-5-oxo-4,5-dihydropyrano[3,2-c]chromene-3-carbonitrile, F19

Off White solid; mp 256–258 °C; 93% yield; IR $\nu_{\max}$: 3300 cm^{-1} $\nu_{(\text{NH}_2)}$, 2100 cm^{-1} $\nu_{(\text{CN})}$, 1712 cm^{-1} $\nu_{(\text{C}=\text{O})}$; ^1H NMR (DMSO- d_6 , 400 MHz) δ : 8.55–8.54 (m, 1H), 8.12–8.11 (m, 1H), 7.77 (d, $J = 8.8\text{ Hz}$, 1H), 7.57–7.54 (m, 1H), 7.50–7.48 (m, 1H), 7.42–7.38 (m, 3H), 7.09–7.06 (m, 1H), 7.04–7.03 (m, 1H), 4.70 (s, 1H), 3.84 (s, 3H), 2.35 (s, 3H); ^{13}C NMR (DMSO- d_6 , 100 MHz) δ : 163.7, 160.3, 159.9, 158.4, 154.8, 157.6, 150.4, 145.5, 138.6, 137.4, 137.7, 134.0, 132.6, 131.4, 130.6, 124.4, 123.5, 119.5, 114.6, 114.4, 113.4, 106.3, 56.7, 56.6, 31.6, 19.4; LCMS (ESI): m/z Calcd for $C_{26}H_{17}ClFN_3O_4$ $[M + H]^+$: 490.0969; Found: 489.9090.

2-amino-4-(3-(6-fluoro-5-methylpyridin-3-yl)-4-methoxyphenyl)-5-oxo-4,5-dihydro-2H-chromene-3-carbonitrile, F20

Yellow solid; mp 198–200 °C; 94% yield; IR ν_{max} : 3300 cm^{-1} $\nu_{\text{(NH}_2\text{)}}$, 2100 cm^{-1} $\nu_{\text{(CN)}}$, 1706 cm^{-1} $\nu_{\text{(C=O)}}$; ^1H NMR (DMSO- d_6 , 400 MHz) δ : 8.02–7.82 (m, 3H), 7.67–7.63 (m, 1H), 7.45–7.35 (m, 4H), 7.25–7.21 (m, 2H), 7.04 (d, J = 8 Hz, 1H), 4.46 (s, 1H), 3.72 (s, 3H), 2.22 (s, 3H); ^{13}C NMR (DMSO- d_6 , 100 MHz) δ : 162.2, 160.4, 160.1, 158.5, 155.7, 153.7, 152.6, 144.7, 143.2, 136.2, 133.9, 132.4, 130.1, 129.4, 125.5, 124.7, 122.9, 119.8, 118.9, 116.9, 113.5, 112.3, 58.4, 56.1, 36.6, 14.4; LCMS (ESI): m/z Calcd for $\text{C}_{26}\text{H}_{18}\text{FN}_3\text{O}_4$ $[\text{M} + \text{H}]^+$: 456.1359; Found: 456.1374.

2-amino-4-(3-(6-fluoro-5-methylpyridin-3-yl)-4-methoxyphenyl)-8-methoxy-5-oxo-4,5-dihydro-2H-chromene-3-carbonitrile, F21

Yellow solid; mp 238–240 °C; 89% yield; IR ν_{max} : 3300 cm^{-1} $\nu_{\text{(NH}_2\text{)}}$, 2100 cm^{-1} $\nu_{\text{(CN)}}$, 1711 cm^{-1} $\nu_{\text{(C=O)}}$; ^1H NMR (DMSO- d_6 , 400 MHz) δ : 8.02 (s, 1H), 7.83 (d, J = 8.8 Hz, 1H), 7.73 (d, J = 8.8 Hz, 1H), 7.32 (s, 2H), 7.24–7.19 (m, 2H), 7.05–7.00 (m, 2H), 6.97 (s, 1H), 4.41 (s, 1H), 3.81 (s, 3H), 3.72 (s, 3H), 2.15 (s, 3H); ^{13}C NMR (DMSO- d_6 , 100 MHz) δ : 163.5, 162.2, 160.4, 159.9, 158.5, 155.7, 154.5, 154.1, 144.7, 143.2, 136.4, 132.5, 132.4, 129.9, 125.4, 124, 119.8, 118.9, 113.1, 112.3, 106.4, 101.2, 58.4, 56.5, 56.1, 36.4, 14.3; LCMS (ESI): m/z Calcd for $\text{C}_{27}\text{H}_{20}\text{FN}_3\text{O}_5$ $[\text{M} + \text{H}]^+$: 486.1465; Found: 485.9829.

2-amino-8-methoxy-4-(6-methoxy-2'-methyl-[1,1'-biphenyl]-3-yl)-5-oxo-4,5-dihydro-2H-chromene-3-carbonitrile, F22

Off White solid; mp 250–252 °C; 94% yield; IR ν_{max} : 3300 cm^{-1} $\nu_{\text{(NH}_2\text{)}}$, 2100 cm^{-1} $\nu_{\text{(CN)}}$, 1711 cm^{-1} $\nu_{\text{(C=O)}}$; ^1H NMR (DMSO- d_6 , 400 MHz) δ : 7.72 (d, J = 9.2 Hz, 1H), 7.30 (s, 2H), 7.19–7.13 (m, 4H), 7.04–6.98 (m, 4H), 6.92 (d, J = 2 Hz, 1H), 4.38 (s, 1H), 3.81 (s, 3H), 3.64 (s, 3H), 1.98 (s, 3H); ^{13}C NMR (DMSO- d_6 , 100 MHz) δ : 163.5, 160.4, 158.4, 155.6, 154.0, 153.9, 138.6, 136.5, 135.7, 130.3, 130.2, 130.0, 128.2, 127.6, 125.8, 123.9, 119.8, 113.2, 111.7, 106.4, 101.2, 101.6, 58.6, 56.5, 55.7, 36.4, 20.1; LCMS (ESI): m/z Calcd for $\text{C}_{28}\text{H}_{22}\text{N}_2\text{O}_5$ $[\text{M} + \text{H}]^+$: 467.1607; Found: 467.1624.

2-amino-9-fluoro-4-(6-methoxy-2'-methyl-[1,1'-biphenyl]-3-yl)-5-oxo-4,5-dihydro-2H-chromene-3-carbonitrile, F23

Off White solid; mp 261–262 °C; 88% yield; IR ν_{max} : 3300 cm^{-1} $\nu_{\text{(NH}_2\text{)}}$, 2100 cm^{-1} $\nu_{\text{(CN)}}$, 1702 cm^{-1} $\nu_{\text{(C=O)}}$; ^1H NMR (DMSO- d_6 , 400 MHz) δ : 7.61–7.58 (m, 1H), 7.56–7.45 (m, 2H), 7.32 (s, 2H), 7.22–7.11 (m, 4H), 7.04–6.95 (m, 3H), 4.44 (s, 3H), 3.64 (s, 1H), 1.98 (s, 3H); ^{13}C NMR (DMSO- d_6 , 100 MHz) δ : 159.9, 158.3, 157.4, 155.7, 152.9, 148.9, 138.6, 136.4, 135.2, 130.4, 130.2, 130.1, 129.9, 128.3, 127.5, 125.8, 120.7, 120.4, 119.5, 119.2, 119.1, 114.6, 114.5, 111.7, 108.7, 108.5, 105.3, 58.5, 55.7, 36.6, 20.1; LCMS (ESI): m/z Calcd for $\text{C}_{27}\text{H}_{19}\text{FN}_2\text{O}_4$ $[\text{M} + \text{H}]^+$: 455.1407; Found: 455.1434.

Density functional theory (DFT) calculations

Molecular DFT investigations were carried out with Gaussian09 software.⁷⁴ The Gaussview 5 program package was employed for molecular building and visualization. The molecular geometry of the molecules was optimized at the B3LYP level using a 6-311++G (d,p) basis set.⁷⁵

Cell line procurement and culture

MCF-7 breast cancer cells (Accession Numbers: ATCC-HTB-22) were obtained from Procell Life Science and Technology Co., LTD, China. These cells were cultured in Minimum Essential Medium (MEM) or Leibovitz's L-15 medium, each supplemented with 2% FBS, providing essential nutrients and growth factors to support cell proliferation. The cells were incubated and cultured in 5% CO_2 at 37 °C, ensuring optimal conditions for growth and viability.

Compound preparation

The compounds of interest were initially dissolved in dimethyl sulfoxide (DMSO) to create stock solutions. These stock solutions were subsequently diluted with the appropriate cell culture medium to achieve the desired final concentrations. The use of DMSO as a solvent ensured the complete dissolution of the compounds while maintaining their stability and activity.

Cell seeding and treatment

A total of 4×10^3 MCF-7 cells per well were seeded into 96-well plates and allowed to adhere and grow overnight. This overnight incubation ensured that the cells reached an optimal confluency before treatment. Following this, the cells were exposed to the compounds at a range of concentrations (0, 0.01, 0.1, 10, 100, and 1000 μ M) for 72 h. The wide range of concentrations allowed for the determination of the dose-response relationship and the calculation of IC₅₀ values, which indicate the concentration at which 50% of the cells are inhibited.

Assessment of cell viability

The inhibitory effects of the compounds on MCF-7 cell viability were assessed using the Alamar Blue reagent. This reagent is a cell viability indicator that incorporates resazurin, a nontoxic, cell-permeable dye that is reduced to resorufin by metabolically active cells. The reduction of resazurin to resorufin results in a fluorescent signal that can be quantitatively measured. The fluorescence intensity is directly proportional to the number of viable cells, providing an accurate measure of cell viability and proliferation. The fluorescence readings were taken using a microplate reader at an appropriate excitation/emission wavelength (typically 570/585 nm). The data obtained were analyzed to determine the inhibitory effects of the compounds on MCF-7 cells, with a focus on identifying potential therapeutic candidates that exhibit significant anti-proliferative activity. By utilizing these comprehensive and scientifically rigorous methodologies, we ensured the reliability and reproducibility of the experimental results, thereby contributing valuable insights into the efficacy of novel compounds against breast cancer cells.

Bioinformatic analysis

Molecular docking simulations between the human intestinal trefoil factor (TFF3) and the AMPC optimized ligands (**F8**, **F10**, **F12** and **F13**) were conducted using AutoDock Software Tools (ADT) version 1.5.7.^{5,76} The X-ray crystal structure of TFF3 (PDB ID: 1PE3^{5,76}) was sourced from the RCSB Protein Data Bank (<https://www.rcsb.org/>). Preparation of the Receptor: The TFF3 structure was prepared for docking by removing heteroatoms and water molecules to ensure a clean receptor environment. Polar hydrogen atoms were added to the structure, and Kollman charges were applied to facilitate accurate electrostatic interactions during docking. Docking Simulations: The docking grid was configured to encompass the entire TFF3 receptor, with cell dimensions set to $100 \times 100 \times 85$ Å, ensuring comprehensive coverage of potential binding sites. For each ligand, docking simulations were performed to predict their binding affinities and interaction modes with TFF3.

The binding energies of the ligands with the TFF3 receptor (PDB ID: 1PE3) were calculated, and the docking conformations were analyzed. The conformation with the lowest binding energy within the largest cluster was selected as the representative model, indicating the most likely binding mode of the ligand. Visualization and Analysis: The docking interactions were visualized and further analyzed using Maestro version 2020.4. This software enabled a detailed examination of the ligand-receptor interactions, providing insights into the binding mechanisms and potential efficacy of the ligands. By utilizing this rigorous methodology, we ensured accurate and reliable docking simulations, contributing to a deeper understanding of the interaction between TFF3 and the AMPC optimized ligands.

Western blot analysis

MCF-7 cells were exposed to compounds; the cells were subsequently collected, and the lysates were extracted. Cells were treated with various concentrations. After treatment, cell lysates were prepared to analyze the expression of proteins. The cells were lysed using a lysis buffer containing protease and phosphatase inhibitors to ensure the stability and integrity of the proteins. The total protein content from these lysates was quantified using the Bicinchoninic Acid (BCA) assay. This colorimetric assay involves the reduction of Cu^{2+} to Cu^{1+} by proteins in an alkaline medium, followed by the formation of a purple-colored complex with BCA, which is measured spectrophotometrically. SDS-PAGE and Western Blotting: For the analysis, 50 μg of total protein from each sample was loaded onto a 12% SDS-PAGE gel. The gel electrophoresis was performed at 100 V for two hours to ensure proper separation of the proteins based on their molecular weights. Post-electrophoresis, the proteins were transferred onto a PVDF or nitrocellulose membrane using a semidry transfer apparatus. This method allows for efficient and rapid protein transfer, which is crucial for subsequent detection steps. The membranes were then blocked with 5% skimmed milk in TBST buffer (Tris-buffered saline with 0.1% Tween 20) for one hour to prevent nonspecific binding of antibodies. Antibody Incubation and Detection: The blocked membranes were washed three times with TBST and incubated overnight at 4°C with primary antibodies specific to TFF3 and beta-actin (internal control) relevant downstream targets. Following primary antibody incubation, the membranes were washed again three times with TBST and incubated with HRP-conjugated secondary antibodies for two hours at room temperature.

Enhanced chemiluminescence (ECL) detection was employed to visualize the protein bands. The ECL reagent, mixed in a 1:1 ratio of reagent 1 and reagent 2, was applied to the membranes. The chemiluminescent signal was detected using a Chemi-UV imaging system in a dark room to ensure optimal band visualization (BIO-RAD, USA).

Conclusions

In summary, this study demonstrates that nano- TiO_2 -mediated synthesis offers an efficient, sustainable, and scalable approach for the preparation of biologically active chromene-3-carbonitrile derivatives. Thermodynamic free energy analysis confirmed the feasibility of the proposed synthetic pathway, supporting the robustness of the catalytic strategy. Biological evaluation revealed that four lead compounds (F8, F10, F12, and F13) exhibited potent cytotoxic activity against MCF-7 breast cancer cells, with IC_{50} values of 0.75, 1.70, 0.68, and 0.52 μM , respectively. These values fall within or below the low-micromolar range commonly reported for chromene-based anticancer agents, highlighting their strength as promising lead scaffolds. Importantly, molecular docking analyses demonstrated strong binding interactions with TFF3, while Western blot results confirmed downregulation of TFF3 protein expression in treated cells. Together, these findings provide mechanistic evidence that the observed cytotoxicity is target-directed through TFF3 inhibition rather than resulting solely from nonspecific cytotoxic effects. Given the established role of TFF3 in tumor progression and endocrine resistance in breast cancer, the identification of chromene-3-carbonitrile derivatives as TFF3-targeted inhibitors expands the currently limited repertoire of small-molecule modulators of this oncogenic protein. The integration of green nanocatalytic synthesis with *in vitro* and *in silico* validation further strengthens the translational relevance of this work.⁵⁶

Quantitative comparison & significance (brief)

Our best lead (F13, $\text{IC}_{50} = 0.52 \mu\text{M}$) is in the sub-micromolar range, which is stronger than many published chromene derivatives that typically report single-digit to low-micromolar IC_{50} against MCF-7 (see Abdelall;⁵¹ Alneyadi;⁵² other chromene series). This indicates our scaffold is a promising starting point for lead optimization.

Future directions

Future investigations should focus on systematic structure–activity relationship (SAR) optimization to enhance potency and selectivity, detailed mechanistic studies to delineate downstream pathway modulation, pharmacokinetic profiling, toxicity evaluation, and *in vivo* efficacy studies in relevant breast cancer models. These steps are essential to validate the therapeutic potential of this new chemical scaffold and to advance it toward preclinical development. For instance, studies should include *in vivo* studies to evaluate the efficacy and safety of chromene-3-carbonitrile analogs in animal models. This will provide critical insights into their therapeutic potential and pharmacokinetics. Detailed mechanistic studies are needed to further elucidate how chromene-3-carbonitrile analogs inhibit TFF3 and affect the downstream signaling pathways in breast cancer cells. Investigate the potential of combining chromene-3-carbonitrile analogs with existing chemotherapy or targeted therapy agents to enhance therapeutic efficacy and overcome resistance mechanisms in breast cancer treatment. Comprehensive studies on the toxicity and bioavailability of these novel compounds are essential. This includes assessing their off-target effects, metabolic stability, and ability to reach and maintain therapeutic concentrations in tumor tissues. Extend the analysis to other cancer cell lines and primary patient-derived tumor cells to evaluate the broader spectrum of anti-cancer activity of chromene-3-carbonitrile analogs. Further optimize the use of nano-TiO₂ particles in the synthesis process to enhance the yield, purity, and scalability of chromene-3-carbonitrile analogs. Investigate the potential of other nanomaterials to improve the efficiency and environmental sustainability of the synthesis process.

Ethics approval

This study does not involve any animal or human experimental models. Hence, ethical approval is not required.

Author's contributions

CRedit: **Dukanya Dukanya**: Conceptualization, Data curation, Writing – review & editing; **Rashmi Sivakumar**: Conceptualization; **Narasimha M. Beeraka**: Conceptualization, Formal analysis, Investigation, Methodology, Visualization, Writing – review & editing; **Hosadurga Kumar Keerthy**: Conceptualization; **Keerthikumara Venkatesha**: Conceptualization; **Shreeja Basappa**: Conceptualization; **Vladimir N. Nikolenko**: Conceptualization; **Kanchugarakoppal S Rangappa**: Conceptualization; **Andreas Bender**: Conceptualization; **Basappa Basappa**: Conceptualization, Formal analysis, Writing – original draft.

Disclosure statement

No potential conflict of interest was reported by the authors.

Declaration of generative AI and AI-assisted technologies in the writing process

The authors did not use any AI technology to execute this work.

Funding

The authors thank the supporting staff of the University. This work was supported by Vision Group on Science and Technology (CESEM), and the Khalifa University of Science, Technology and Research (Government of Karnataka).

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