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Synthesis and antiproliferative activity of RITA and its analogs

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Efficient synthesis of RITA and its analogues: derivation of analogues with improved antiproliferative activity via modulation of p53/miR-34a pathway†

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A novel approach to synthesize RITA by practical palladium-catalyzed C–C bond-forming Suzuki reactions at room temperature was developed, which was used for deriving a series of substituted tricyclic α-heteroaryl (furan/thiophene) analogues of RITA under mild conditions. These novel analogues showed notable antiproliferative activity against cancer cell lines with wild-type p53 (*i.e.*, HCT116, A549, MCF-7 and K562), but much less activity in HCT116/p53^{−/−} cells. In particular, compound **1f** demonstrated promising antiproliferative activity compared to RITA, with IC₅₀ = 28 nM in MCF-7 vs. 54 nM for RITA, and cancer cell selectivity. Compound **1f** markedly activated p53 in HCT116 cells at 100 nM, triggering apoptosis. Importantly, we found that both RITA and compound **1f** induced G₀/G₁ cell cycle arrest by up-regulating miR-34a, which in turn down-regulated the expression of cell cycle-related proteins CDK4 and E2F1. In summary, this study reports an effective synthetic approach for RITA and its analogues, and elucidates a novel antiproliferative mechanism of these compounds.

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Introduction

Tumor suppressor p53 acts as a central transcription factor in the regulation of cell cycle, apoptosis, DNA repair, and senescence induced by diverse stress.¹ MDM2 (murine double minute 2), an E3 ubiquitin ligase, is a major negative regulator of p53, which binds to the N-terminus of p53 to inhibit its transcriptional activity and to trigger proteasome-mediated degradation.² In human tumors retaining wild-type p53 (p53wt), the activity of p53 can be restored by blocking the p53–HDM2 (human double minute 2) complex. Several classes of small molecules have been developed to serve as p53-

MDM2 inhibitors (Fig. 1), such as AM-8553,³ Nutlin3a,⁴ MI-43,⁵ RITA ((5,5'-(furan-2,5-diyl)bis(thiophene-5,2-diyl))-dimethanol),⁶ etc. Among these compounds, low-molecular-weight RITA, which was identified in the NCI Anticancer Drug Screen, shows promising activity in suppressing tumor cell growth through p53-mediated apoptosis and cell cycle arrest in multiple cancer cell lines both *in vitro* and *in vivo*.⁶

The detailed molecular mechanisms of RITA-induced cancer cell growth inhibition remain to be fully elucidated. Selivanova and co-workers found that RITA could bind to the N-terminal domain of p53 with a high affinity (K_d = 1.5 nM) and prevent p53–HDM2 interaction and ubiquitin-mediated degradation.⁷ However, a controversial report indicated that p53 may not bind to HDM2.⁸ Nevertheless, it is well accepted that RITA abrogates key oncogenic pathways by activating p53.⁹ For instance, RITA triggers p53-mediated transcription of proapoptotic genes (*e.g.* Puma, Noxa, and Bax) and unleashes the transcriptional suppression of antiapoptotic proteins (*e.g.* Mcl-1, Bcl-2, MAP4, survivin). RITA also down-regulates the expression of oncogenic proteins (*i.e.*, c-Myc, cyclin E, and β-catenin) and blocks the Akt pathway.¹⁰ In addition, activation of p53 by RITA down-regulates the expression of p53 target genes (p21, hnRNPK *etc.*), HIF-1α (Hypoxia-Inducible Factor 1α) and VEGF (vascular endothelial growth factor).¹⁰ Interestingly, our study revealed that RITA might induce cell cycle arrest through up-regulation of miR-34a.

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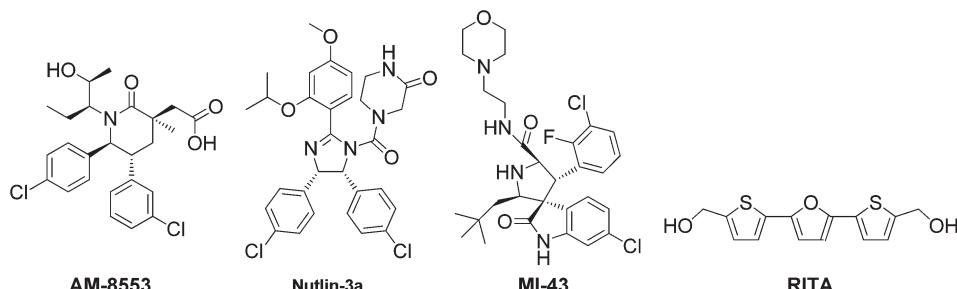
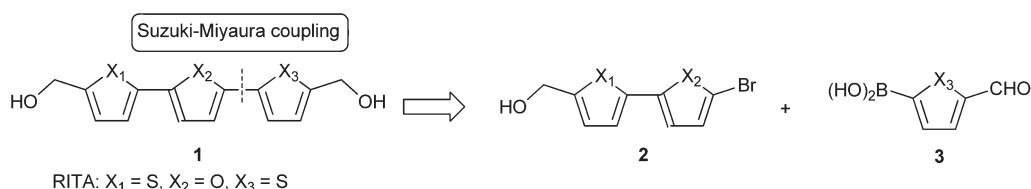
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**Fig. 1** p53-MDM2 inhibitors.**Scheme 1** Retrosynthetic analysis of RITA and its analogues.

MicroRNAs (miRs) are small non-coding RNAs 18–25 nucleotides in length that regulate gene expression during various crucial cell processes, such as apoptosis, differentiation and development.¹¹ Alterations in miRNA expression have been observed in a variety of human tumors.¹² Functional studies indicate that miRNAs act as either tumor suppressors or oncogenes. MiR-34a is a direct downstream effector of p53 and is involved in p53-induced cell senescence and apoptotic pathways.¹³ The level of miR-34a is down-regulated in diverse tumors compared with normal tissue counterparts.¹⁴ The restoration of miR-34a expression resulted in tumor cell apoptosis, cell-cycle arrest or senescence, indicating that miR-34a acts as an important tumor suppressor in the p53 network during cancer development.¹⁵

RITA is a small molecule with promising antiproliferative activity, but the efficiency of current synthetic methods is limited. Chang's group¹⁶ firstly developed a synthetic method to generate RITA through a four-step reaction, including formaldehyde protection and deprotection, Stille coupling reaction and sodium borohydride reduction. The overall yield of RITA is only about 37%. R. Danae *et al.*¹⁷ reported a synthetic approach to RITA's precursor, the dialdehyde derivative, utilizing a one pot Stille coupling (yield: 35%) and formylation (yield: 24%, at -78 °C) reaction. However, the expensive and toxic reagents (the toxic chlorotributyltin and expensive 2,5-dibromofuran), harsh reaction conditions (to produce tributylstannanyl-thiophene-2-carbaldehyde for the Stille reaction at -78 °C) and the low yield of products make these methods unsuitable for large-scale production. Therefore, it is necessary to develop a more practical method to synthesize RITA.

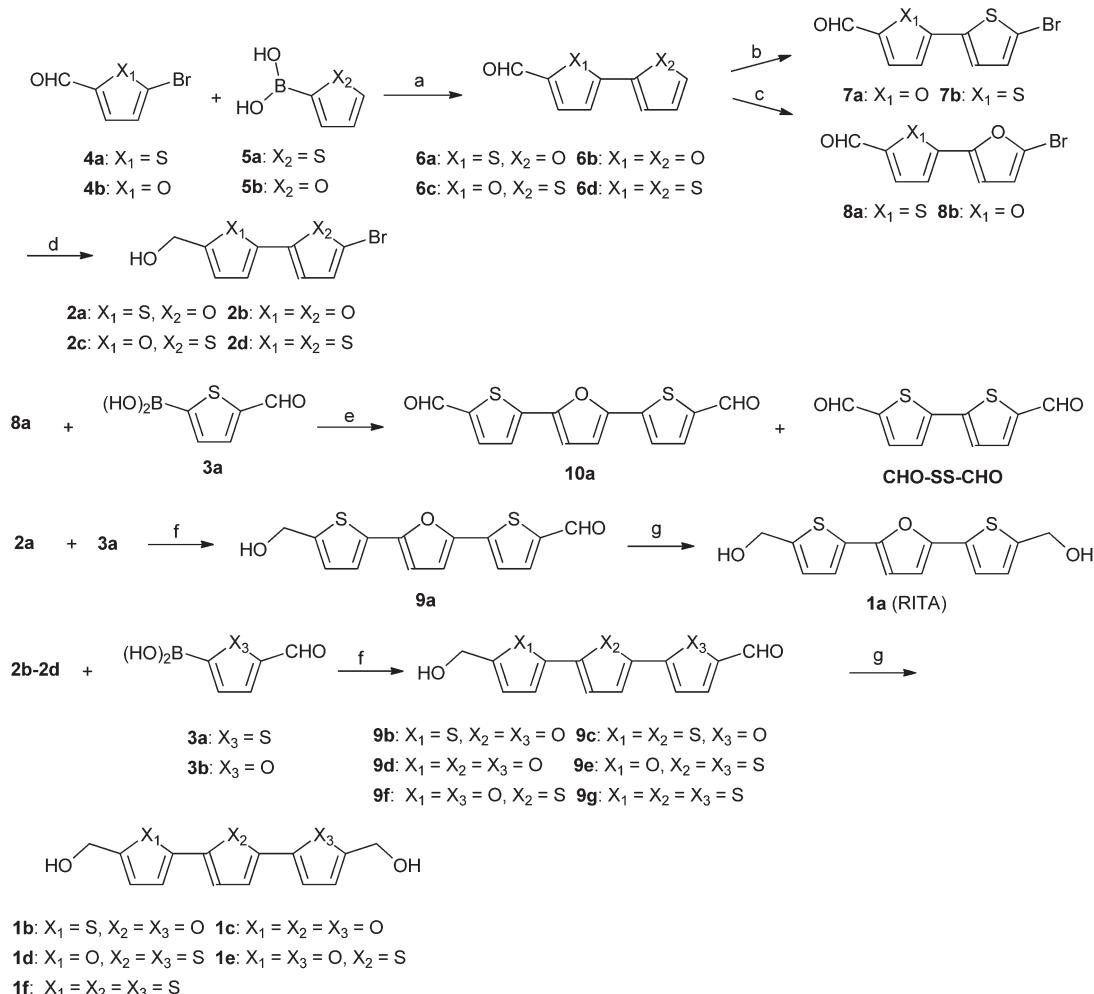
In this study, we developed a novel, effective synthetic route to RITA, which was used for deriving a series of RITA analogues both for testing our method and for discovering new antiproliferative agents, particularly those with potent activity,

and we further found a p53/miR-34a mediated antiproliferative pathway induced by RITA and its analogues.

Results and discussion

A novel, effective synthetic route to RITA and its analogues

Retrosynthetic analysis indicated that a Suzuki–Miyaura reaction could be used to synthesize RITA, and this reaction was used to prepare tricyclic α-heteroaryl thiophene analogues of RITA (Scheme 1).¹⁸ As shown in Scheme 2, a 5-step route was carried out to synthesize RITA. The intermediates **6** were synthesized by Suzuki coupling of the commercially available compounds **4** and **5** at room temperature using Pd(OAc)₂–X-Phos as catalysts in CH₃CN–H₂O under argon atmosphere. Bromination of substituted **6** with NBS at -23 °C provided respectively **7** and **8** in good yields.^{18,19} C–C bond-forming Suzuki reaction of **8a** with commercially available **3a** in the presence of Pd(PPh₃)₄–Na₂CO₃²⁰ in dioxane–H₂O afforded the precursor **10a** in 16% yield (entry 1). Homocoupling of heteroaryl boronic acids (**CHO-SS-CHO**) occurred as the main process, and separation of **10a** from the homocoupling product was very difficult due to their very similar *R*_f values. Different reaction conditions including ligands, bases, and the source of Pd in the cross-coupling reaction were investigated, all of which have been successfully performed in the literature. As shown in Table 1, the use of Pd(PPh₃)₄–K₂CO₃ as catalyst in DME (1,2-dimethoxyethane) at 80 °C under argon atmosphere¹⁸ only afforded **10a** in 12% yield (entry 2). Only trace or no product was detected when the reaction was carried out using Pd(PPh₃)₂Cl₂–K₂CO₃ as catalysts in DME–CH₂Cl₂ at 80 °C.²¹ Pd(OAc)₂–PPh₃–Na₂CO₃ in DME²² and Pd(OAc)₂–K₂CO₃–TBAB (tetrabutylammonium bromide) in H₂O²³ under argon atmosphere (entries 3–5) were similarly unsuccessful.



Scheme 2 Synthesis of **1**. Reagents and conditions: (a) Pd(OAc)₂ (1 mol%), K₂CO₃, X-Phos (2 mol%), CH₃CN-H₂O (1.5 : 1), rt, 8–12 h; (b) NBS, DMF, -23 °C, 4 h; (c) NBS, BPO, toluene, -23 °C, 4 h; (d) NaBH₄, CH₂Cl₂-CH₃OH, 0 °C, 30 min; (e) conditions in Table 1; (f) Pd(OAc)₂ (1 mol%), K₂CO₃, X-Phos (2 mol%), CH₃CN-H₂O (1.5 : 1), rt, 24 h; (g) NaBH₄, CH₂Cl₂-CH₃OH, 0 °C, 30 min.

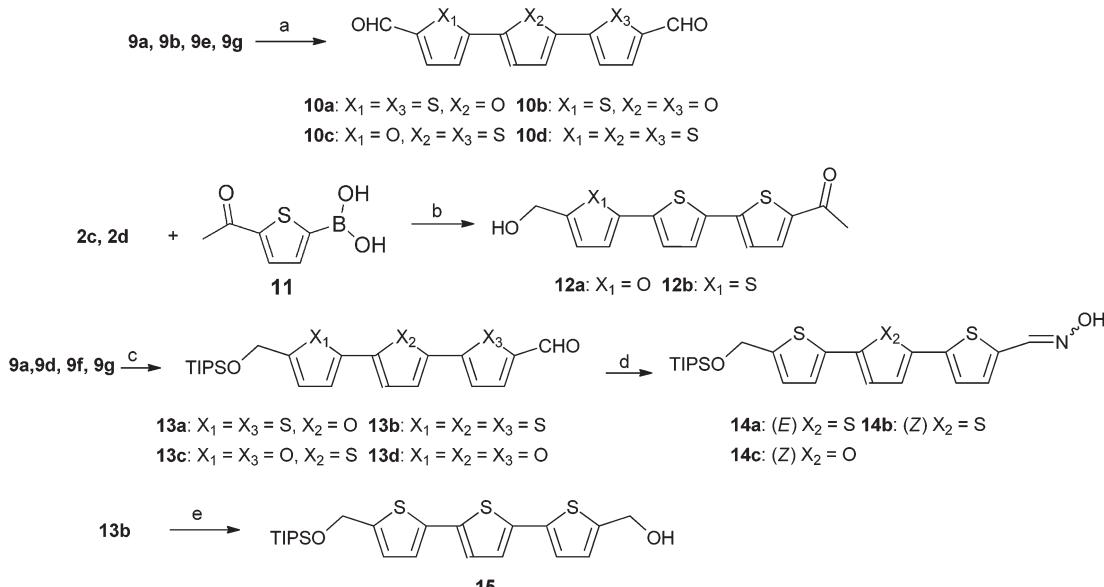
Table 1 Optimization of reaction conditions for the Suzuki coupling

| Entry | Substrates | Solvent | [Pd]/Ligand | Base | T (°C)/t [h] | Yield ^a (%) |
|-------|----------------|-------------------------------------|--|---------------------------------|--------------|------------------------|
| 1 | 8a + 3a | Dioxane-H ₂ O | Pd(PPh ₃) ₄ | Na ₂ CO ₃ | 100/12 | 16 |
| 2 | 8a + 3a | DME | Pd(PPh ₃) ₄ | K ₂ CO ₃ | 80/20 | 12 |
| 3 | 8a + 3a | DME-CH ₂ Cl ₂ | Pd(PPh ₃) ₂ Cl ₂ | K ₂ CO ₃ | 80/2 | Trace |
| 4 | 8a + 3a | H ₂ O | Pd(OAc) ₂ | K ₂ CO ₃ | rt/2 | 0 |
| 5 | 8a + 3a | DME | Pd(OAc) ₂ /PPh ₃ | Na ₂ CO ₃ | refl/4 | Trace |
| 6 | 8a + 3a | CH ₃ CN-H ₂ O | Pd(OAc) ₂ /S-Phos | K ₂ CO ₃ | refl/24 | 24 |
| 7 | 8a + 3a | CH ₃ CN-H ₂ O | Pd(OAc) ₂ /X-Phos | K ₂ CO ₃ | refl/24 | 28 |
| 8 | 2a + 3a | CH ₃ CN-H ₂ O | Pd(OAc) ₂ /X-Phos | K ₂ CO ₃ | refl/24 | 62 |
| 9 | 2a + 3a | CH ₃ CN-H ₂ O | Pd(OAc) ₂ /X-Phos | K ₂ CO ₃ | rt/24 | 64 |

^a Isolated yield.

The reaction yield improved slightly when Pd(OAc)₂-S-Phos (2-dicyclohexylphosphino-2',6'-dimethoxybiphenyl) or Pd(OAc)₂-X-Phos (2-dicyclohexyl-phosphino-2',4',6'-triisopropylbiphenyl) were used as catalysts in refluxing CH₃CN-H₂O under argon atmosphere (entries 6–7), but the main product was still CHO-SS-CHO. In order to facilitate the separation of **10a** from

the homocoupling product, reduction of **8a** with NaBH₄ in CH₂Cl₂-CH₃OH at 0 °C gave **2a**, which was selected to react with **3a** through Suzuki reaction to furnish **9a** using Pd(OAc)₂-X-Phos in refluxing CH₃CN-H₂O within 24 h under argon atmosphere. Surprisingly, the desired **9a** was obtained in 62% yield (entry 9), with no CHO-SS-CHO detected (Scheme 2). To



Scheme 3 Synthesis of RITA analogues. Reagents and conditions: (a) Oxone, TEMPO, TBAB, CH_2Cl_2 -acetone, rt, 1–3 h; (b) $\text{Pd}(\text{OAc})_2$ (1 mol%), K_2CO_3 , X-Phos (2 mol%), $\text{CH}_3\text{CN}-\text{H}_2\text{O}$, rt, 24 h; (c) TIPSCl, imidazole, dry THF, rt, 24 h; (d) $\text{NH}_2\text{OH}\cdot\text{HCl}$, NaOAc , MeOH , reflux, 2 h; (e) NaBH_4 , $\text{CH}_2\text{Cl}_2-\text{CH}_3\text{OH}$, 0 °C, 30 min.

our delight, when this reaction was carried out at room temperature, the yield increased slightly to 64%. In addition, when the amount of the substrate **2a** was increased from 3.64 mmol (0.94 g) to 36.4 mmol (9.4 g) and the amount of **3a** and $\text{Pd}(\text{OAc})_2\text{-X-Phos}$ were increased correspondingly, the desired product was isolated in 64–68% yields. Reduction of **9a** with NaBH_4 furnished the target molecule **1a** (RITA) in 5 steps and an overall yield of 53%, which is higher than that in the reported literature (37%¹⁶). Compared to the reported synthetic routes, this novel and practical approach to RITA is suitable for large-scale production with cheap commercially available materials, high yield and mild conditions. Under the optimised conditions, RITA and a series of its analogues **9a–9g**, **1b–1f** were synthesized (Scheme 2). Other RITA derivatives were synthesized as outlined in Scheme 3. Oxidation of **9** with Oxone-TEMPO at room temperature in the presence of TBAB furnished the corresponding dialdehyde **10**.²⁴ Suzuki reaction of **2** with **11** in the presence of $\text{Pd}(\text{OAc})_2\text{-X-Phos}$ in $\text{CH}_3\text{CN}-\text{H}_2\text{O}$ at room temperature within 24 h under argon atmosphere afforded the tricyclic heteroaryl compound **12**. **9** was protected with the triisopropylsilyl (TIPS) group to give **13**,²⁵ which was treated with $\text{NH}_2\text{OH}\cdot\text{HCl}$, AcONa (1.5 equiv) in refluxing CH_3OH to furnish oximes **14**. Reduction of **13b** with NaBH_4 afforded **15**.

Inhibition of cell growth by new RITA analogues

Using MTT assays, we measured the antiproliferative activity of RITA and its analogues in HCT116/*p53*^{wt}, MCF-7, A549, K562 and HCT116/*p53*^{-/-} cells. As shown in Table 2, some analogues such as compound **1f** exhibited better activity than RITA, with IC_{50} values of 28 nM in MCF-7 cells and 100 nM in HCT116 cells (Fig. 2A). The data from this series of RITA analogues provided us with the following information on the

structure–activity relationships (SAR): (1) these analogues possess notable activity against the cancer cell lines with wild-type p53 (HCT116, A549, MCF-7, and K562), but not in HCT116/*p53*^{-/-}; (2) for analogues with the same side chains, compounds containing sulfur atoms at X_1 , X_3 or at X_1 , X_2 , and X_3 possess comparatively stronger antiproliferative activity than the other derivatives, as exhibited by **1a–1f**, **9a–9g**, etc.; (3) for the same combination of X_1 , X_2 , X_3 , analogues with the side chains with two hydroxymethyl groups showed better antiproliferative activity than those with a hydroxymethyl group and an aldehyde group, followed by two aldehyde groups, as demonstrated by RITA, **9a** and **10a**. Furthermore, we also found that if one of the side chains was protected by TIPS, as in the case of compounds **13a** and **15**, this resulted in no antiproliferative activity, suggesting that the hydrophilic group in the side chain may be conducive to the formation of a hydrogen bond with the target, improving pharmacokinetic properties and antitumor activity.

We further measured the cytotoxic activity of these analogues against a normal human colon mucosal epithelial cell line, NCM460. As shown in Table 3, RITA exhibited weak cytotoxicity to NCM460 cells with an inhibition rate of 24.49%, but compound **1f** had much less activity against NCM460, with an inhibition rate of 2.62% at the concentration of 10 μM (100 fold higher than the IC_{50} in colon cancer cells HCT116).

The antiproliferative activity of compound **1f** was confirmed by its capability of suppressing the clonogenic growth of HCT116 cells. At the low concentration of 0.0625 μM , compound **1f** greatly decreased the colony formation of HCT116 cells (Fig. 2B). Taken together, these

Table 2 Antiproliferation activity of compounds against HCT116, MCF-7, A549, K562, HCT116/p53^{-/-} cells

| Compound | HCT116 | MCF-7 | A549 | K562 | HCT116/p53 ^{-/-} |
|------------------|--------------|---------------|--------------|-------------|---------------------------|
| 1a (RITA) | 0.10 ± 0.02 | 0.054 ± 0.010 | 7.30 ± 0.02 | 0.50 ± 0.04 | 16.00 ± 0.19 |
| 1b | 2.10 ± 0.06 | >25 | >25 | >25 | >25 |
| 1c | >25 | >25 | >25 | >25 | >25 |
| 1d | 6.30 ± 0.14 | 3.50 ± 0.08 | 6.90 ± 0.04 | 0.41 ± 0.03 | >25 |
| 1e | 19.40 ± 0.17 | 0.40 ± 0.09 | 11.20 ± 0.06 | 2.79 ± 0.04 | >25 |
| 1f | 0.10 ± 0.01 | 0.028 ± 0.010 | 6.81 ± 0.07 | 0.26 ± 0.08 | 15.90 ± 0.12 |
| 9a | 0.33 ± 0.04 | 0.40 ± 0.05 | 6.90 ± 0.04 | 2.79 ± 0.06 | >25 |
| 9b | 5.21 ± 0.08 | 6.90 ± 0.13 | 5.70 ± 0.05 | 3.12 ± 0.09 | >25 |
| 9c | 5.00 ± 0.11 | 5.70 ± 0.06 | 5.10 ± 0.09 | >25 | >25 |
| 9d | >25 | >25 | >25 | >25 | >25 |
| 9e | >25 | >25 | >25 | >25 | >25 |
| 9f | >25 | >25 | 7.10 ± 0.10 | >25 | >25 |
| 9g | 0.50 ± 0.04 | 0.60 ± 0.03 | 11.40 ± 0.16 | 1.46 ± 0.09 | >25 |
| 10a | 5.50 ± 0.12 | >25 | 11.60 ± 0.08 | >25 | >25 |
| 10b | >25 | >25 | >25 | >25 | >25 |
| 10c | >25 | >25 | >25 | >25 | >25 |
| 10d | 5.50 ± 0.02 | >25 | 12.81 ± 0.08 | >25 | >25 |
| 12a | 3.10 ± 0.07 | >25 | >25 | 2.71 ± 0.20 | >25 |
| 12b | 0.42 ± 0.02 | >25 | >25 | 0.41 ± 0.07 | >25 |
| 13a | >25 | >25 | >25 | >25 | >25 |
| 13b | >25 | >25 | >25 | >25 | >25 |
| 13c | >25 | >25 | >25 | >25 | >25 |
| 13d | >25 | >25 | >25 | >25 | >25 |
| 14a | >25 | >25 | >25 | >25 | >25 |
| 14b | >25 | >25 | >25 | >25 | >25 |
| 14c | >25 | >25 | >25 | >25 | >25 |
| 15 | >25 | >25 | >25 | >25 | >25 |

^a All values are means of three experiments.

data suggest that compound **1f** is a potent anticancer analogue of RITA.

p53-dependent apoptosis induced by RITA and compound **1f**

Compound **1f** was chosen for further studies based on its potent antiproliferative activity with the use of RITA as a control. HCT116 cells were treated with 0.1 µM RITA or compound **1f** for 48 h and then stained with Hoechst 33258 for fluorescence microscopy observations. As shown in Fig. 3A, characteristic morphological changes typical of apoptosis, such as membrane blebbing, chromatin condensation, and the formation of apoptotic bodies, were observed. An annexin-V/PI (propidium iodide) binding assay further confirmed the apoptosis induced by compound **1f** and RITA. As shown in Fig. 3B, the upper-right quadrant (both annexin-V and PI positive) represents the late stage apoptotic cells and the lower-right quadrant (annexin-V positive but PI negative) indicates the early stage apoptotic cells. In RITA-treated HCT116 cells, 30.50% cells were in early apoptosis and 36.60% in late apoptosis, whereas compound **1f** showed similar activity with 22.90% in early apoptosis stage and 44.15% in late apoptosis. These results demonstrate that compound **1f** could efficiently induce HCT116 cell death. To determine the p53-dependence of the apoptosis induced by compound **1f**, we examined the p53 levels in HCT116 cells. As shown in Fig. 4, compound **1f** indeed activated p53 in the cells, indicating that compound **1f** induces apoptosis by triggering p53.

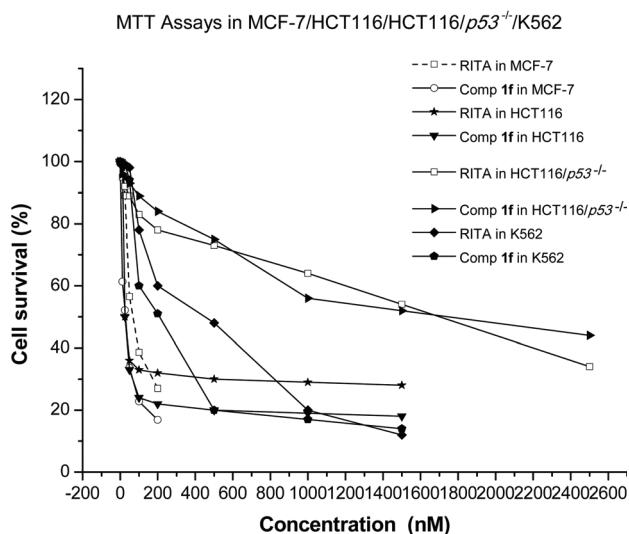
Compound **1f** induces G₀/G₁ cell cycle arrest through up-regulation of miR-34a

MiR-34a is an important target of p53.^{13b} In this study, we further examined the effect of compound **1f** and RITA on miR-34a expression in HCT116 cells using real-time reverse transcription-PCR. As shown in Fig. 5A, the expression of miR-34a was dramatically increased by both compound **1f** and RITA in a time-dependent manner. MiR-34a functions as a key target for p53 in the regulation of cell cycle progression and cell proliferation by down-regulating the expression of cell-cycle related proteins, such as E2F1, CDK4 and MET.^{13a} Our data showed that in response to p53 activation, miR-34a significantly decreased CDK4 and E2F1 protein levels (Fig. 5B), leading to G₀/G₁ cell cycle arrest (Fig. 5C).

Molecular docking

It was established that RITA can bind to the HDM2-p53 transactivation domain binding cleft by computational “blind docking” studies.²⁶ To better understand the interaction between compounds and their targets, molecular docking studies on RITA and compound **1f** were conducted employing the Discovery Studio 3.1/Libdock protocol (Accelrys Software Inc.) and the X-ray structure of HDM2 in complex with the p53 transactivation domain (PDB code: 1YCR).²⁷ Fig. 6 shows that RITA docks into the HDM2-p53 transactivation domain binding cleft. In this binding model, RITA is bound to the hydrophobic HDM2-p53 transactivation domain binding cleft

A



B

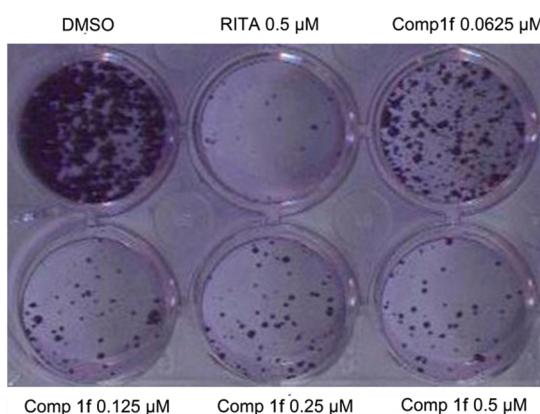


Fig. 2 Antiproliferative activity of RITA and compound **1f**. (A) Effect of RITA and compound **1f** on the viability of HCT116, HCT116/*p53*^{-/-}, K562, MCF-7 cells. (B) Effect of RITA and compound **1f** on colony formation of HCT116 cells.

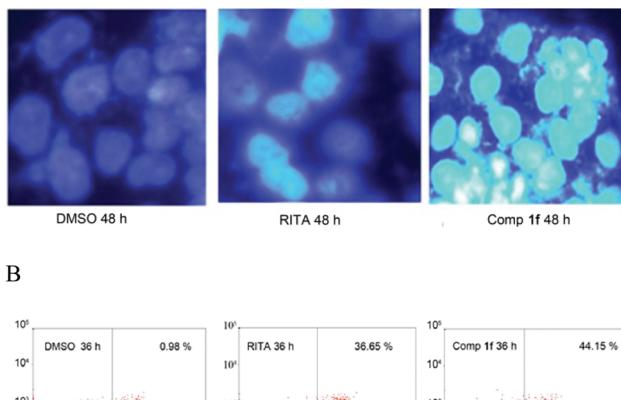
Table 3 Toxicity evaluation of compounds against NCM460 cells^a

| Compound | Inhibition rate (μM) | Compound | Inhibition rate (μM) |
|-----------|----------------------|------------|----------------------|
| 1a | 24.49 ± 0.03% | 9a | 85.73 ± 0.02% |
| 1d | 15.04 ± 0.02% | 9g | 36.76 ± 0.10% |
| 1e | 25.81 ± 0.06% | 12a | 3.33 ± 0.02% |
| 1f | 2.62 ± 0.01% | 12b | 42.57 ± 0.03% |

^a NCM460 cells were treated by compounds at 10 μM for 72 h, and all values are means of three experiments.

and forms three hydrogen bonds with the amino acids Gln72 (O-H···O:2.084 Å), Val93 (O-H···O:2.285 Å), His96 (O-H···O:1.907 Å) (Fig. 6A). Compound **1f** has similar interactions with the HDM2-p53 transactivation domain binding cleft (Fig. 6B), forming a hydrogen bond with Gln72 (O-H···O:2.024 Å) and a π-π interaction between the thiophene ring and His96. In addition, the results of the molecular

A



B

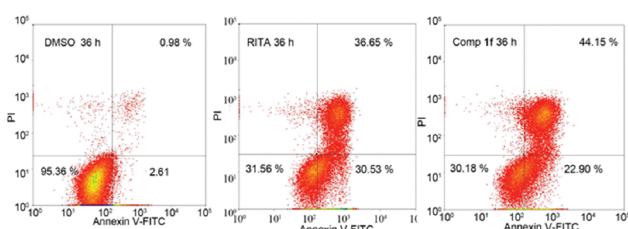


Fig. 3 Apoptosis induced by RITA and compound **1f**. (A) Fluorescent staining of nuclei by Hoechst 33258: H116 cells treated with 0.1 μM RITA or compound **1f** for 48 h; (B) Flow cytometric analysis of phosphatidylserine externalization (Annexin-V binding) and cell membrane integrity (PI staining). HCT116 cells were treated with RITA or compound **1f** at 2 μM for 36 h.

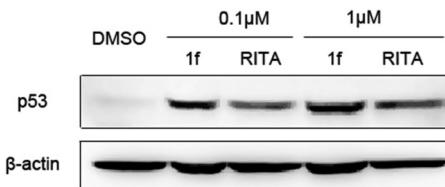


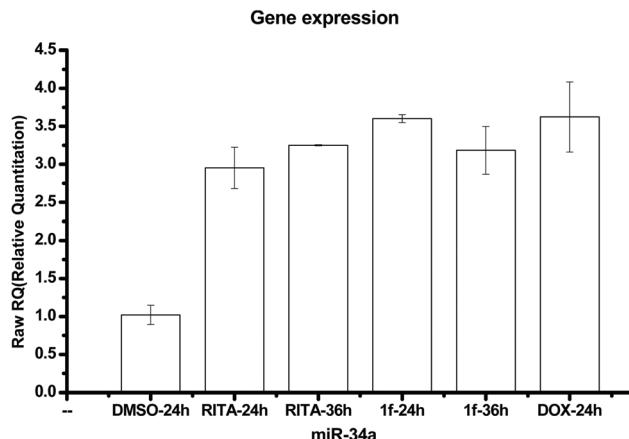
Fig. 4 Effect of RITA and compound **1f** on p53 expression. HCT116 cells were treated with RITA or compound **1f** for 12 h, then cell extracts were subjected to western blot analysis using the indicated antibodies.

dynamics studies also indicated that compound **1f** binds to the target with substantially better affinity compared to RITA, with a $\Delta G = -26.15429$ kcal mol⁻¹ vs. -11.51159 kcal mol⁻¹ for RITA, which explains the improved antitumor activity.

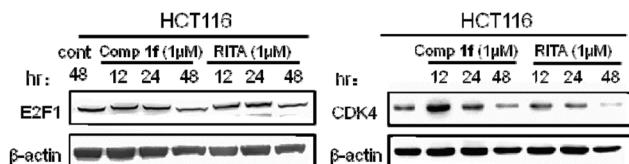
Conclusions

We have developed a novel synthetic method for the preparation of RITA by an efficient and practical palladium-catalyzed C-C bond-forming Suzuki reaction as the key reaction at room temperature with cheap commercially available materials, which provides a valuable and convenient tool to derive a series of substituted tricyclic α-heteroaryl (furan/thiophene) analogues of RITA under mild conditions. Some of the newly derived RITA analogues displayed promising antiproliferative activity in p53 wild-type cells and cancer cell selectivity compared to RITA. Compound **1f** also exhibited weak cytotoxicity to human normal NCM460 cells. The structure-activity relationships (SAR) of these compounds were also analyzed.

A



B



C

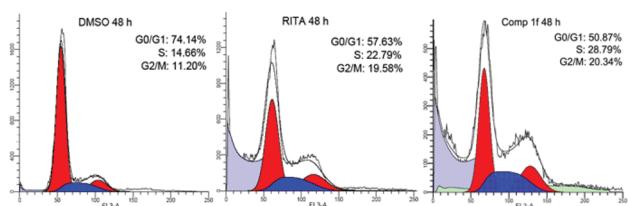


Fig. 5 MiR-34a mediated cell cycle arrest. (A) MiR-34 expression induced by RITA and compound **1f** in HCT116 cells as assessed by Taqman q-RT PCR assay. HCT116 cells were treated with 0.4 μ M RITA or compound **1f** with DMSO and DOX (Doxorubicin hydrochloride) used as controls at the indicated time points. The total RNA (10 ng) extracted from each of the treated cells was used for real-time reverse transcription-PCR (RT-PCR) analysis of miR-34a and RNU66 expression. RNU66 transcripts were used as an internal control. The data represent the average of three independent experiments \pm SD. (B) The effect of RITA and compound **1f** on CDK4 and E2F1 protein levels. HCT116 cells were treated with 1 μ M RITA and compound **1f** for 12 h, 24 h and 48 h, and then cell extracts were subjected to western blot analysis using the indicated antibodies; (C) Analysis of cell cycle phase distribution induced by RITA and compound **1f**. HCT116 cells were treated by 0.25 μ M RITA or compound **1f** for 48 h, and then analyzed by flow cytometry.

The results of molecular docking studies support the view that the antiproliferative activity of RITA and its analogues is partly due to their interactions with the HDM2-p53 transactivation domain binding cleft, providing a clue for further modifications of RITA. Similarly to RITA, compound **1f** markedly activates p53 in HCT116 cells, inducing apoptosis. Both compound **1f** and RITA trigger miR-34a mediated G₀/G₁ cell cycle arrest by down-regulating the expression of cell cycle-related proteins CDK4 and E2F1. In summary, the results of

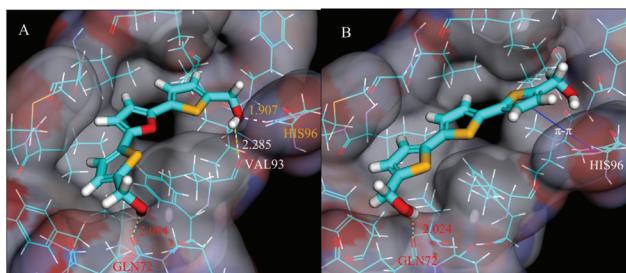


Fig. 6 Molecular docking of RITA and compound **1f** with HDM2. (A) RITA with HDM2; (B) Compound **1f** with HDM2.

this research not only elucidate a novel antiproliferative mechanism for RITA and its analogues, but also provide important information on the synthesis and modifications of RITA and its substituted tricyclic α -heteroaryl (furan/thiophene) analogues.

Experimental

General experimental conditions

Unless otherwise noted, all solvents and reagents were commercially available and used without further purification. ¹H NMR spectra were recorded on a Bruker AVIII-400 spectrometer. Chemical shifts (δ in ppm) were referenced to tetramethylsilane ($\delta = 0$ ppm) in CDCl₃, d₆-DMSO or CD₃OD as an internal standard. ¹³C NMR spectra were obtained by using the same NMR spectrometers, which were calibrated with CDCl₃, d₆-DMSO or CD₃OD. Coupling constants (J) were in hertz (Hz), and signals were designated as follows: s, singlet; d, doublet; t, triplet; m, multiplet, etc. Mass spectra were recorded using a Waters Micromass Q-TOF Premier Mass spectrometer. Thin layer chromatography (TLC) was carried out by using plate silica gel F254. Melting points were determined with a SGW X-4 digital apparatus, and are uncorrected.

Chemistry

GENERAL PROCEDURE FOR PREPARATION OF COMPOUNDS 6A–6D. To a solution of heteroaryl halide **4** (1.0 equiv), boronic acid **5** (1.5 equiv), K₂CO₃ (3.0 equiv), and 2-dicyclohexylphosphino-2',4',6'-triisopropylbiphenyl (X-Phos, 0.02 equiv) in CH₃CN-H₂O (1.5 : 1) was added Pd(OAc)₂ (0.01 equiv). The flask and its contents were put under reduced pressure then backfilled with argon three times. The mixture was stirred at 25 °C for 8 to 12 h under argon atmosphere, then extracted with CH₂Cl₂, and the combined organic layer was washed with brine and dried (Na₂SO₄). The solvent was removed *in vacuo* to afford a crude product, which was purified by flash chromatography (eluting with *n*-hexane-CH₂Cl₂ mixtures).

5-(FURAN-2-YL)THIOPHENE-2-CARBALDEHYDE (**6a**). The general procedure was used with 5-bromothiophene-2-carbaldehyde (**4a**, 1.09 g, 5.71 mmol) and furan-2-ylboronic acid (**5b**, 0.96 g, 8.57 mmol). The product was purified by column chromatography on silica gel (*n*-hexane-CH₂Cl₂, 4 : 1) to provide the title

compound as a light brown solid (0.92 g, 90%). ^1H NMR (400 MHz, CDCl_3): δ 9.88 (s, 1H), 7.69 (d, J = 4.0 Hz, 1H), 7.53–7.47 (m, 1H), 7.32 (d, J = 4.0 Hz, 1H), 6.75 (d, J = 3.4 Hz, 1H), 6.51 (dd, J = 3.4, 1.8 Hz, 1H); ^{13}C NMR (101 MHz, CDCl_3): δ 182.68, 148.27, 143.57, 142.77, 141.57, 137.22, 122.96, 112.37, 108.79.

[2,2'-BIFURAN]-5-CARBALDEHYDE (**6b**). The general procedure was used with 5-bromofuran-2-carbaldehyde (1 g, 5.71 mmol) and furan-2-ylboronic acid (0.96 g, 8.57 mmol). The product was purified by column chromatography on silica gel (*n*-hexane– CH_2Cl_2 , 3 : 1) to provide the title compound as a brown solid (0.73 g, 78%). ^1H NMR (400 MHz, CDCl_3): δ 9.63 (s, 1H), 7.55–7.51 (m, 1H), 7.30 (d, J = 3.8 Hz, 1H), 6.91 (d, J = 3.4 Hz, 1H), 6.74 (d, J = 3.7 Hz, 1H), 6.54 (dd, J = 3.5, 1.8 Hz, 1H); ^{13}C NMR (101 MHz, CDCl_3): δ 177.00, 151.60, 151.44, 144.95, 144.01, 123.42, 112.14, 109.62, 107.42.

5-(THIOPHEN-2-YL)FURAN-2-CARBALDEHYDE (**6c**). The general procedure was used with 5-bromofuran-2-carbaldehyde (1 g, 5.71 mmol) and thiophen-2-ylboronic acid (1.1 mg, 8.57 mmol). The product was purified by column chromatography on silica gel (*n*-hexane– CH_2Cl_2 , 4 : 1) to provide the title compound as a brown solid (0.86 g, 84%). ^1H NMR (400 MHz, CDCl_3): δ 9.60 (s, 1H), 7.52 (d, J = 3.5 Hz, 1H), 7.40 (d, J = 4.9 Hz, 1H), 7.29 (d, J = 3.7 Hz, 1H), 7.10 (t, J = 4.3 Hz, 1H), 6.67 (d, J = 3.6 Hz, 1H); ^{13}C NMR (101 MHz, CDCl_3): δ 176.87, 154.89, 151.55, 131.69, 128.20, 127.53, 126.27, 107.53.

[2,2'-BITHIOPHENE]-5-CARBALDEHYDE (**6d**). The general procedure was used with 5-bromo thiophene-2-carbaldehyde (1.09 g, 5.71 mmol) and thiophen-2-ylboronic acid (1.1 g, 8.57 mmol). The product was purified by column chromatography on silica gel (*n*-hexane– CH_2Cl_2 , 5 : 1) to provide the title compound as a light brown solid (1.04 g, 94%). m.p.: 44–46 °C; ^1H NMR (400 MHz, CDCl_3): δ 9.86 (s, 1H), 7.67 (d, J = 3.9 Hz, 1H), 7.36 (d, J = 4.5 Hz, 2H), 7.25 (s, 1H), 7.10–7.06 (m, 1H); ^{13}C NMR (101 MHz, CDCl_3): δ 182.48, 147.19, 141.84, 137.19, 136.11, 128.38, 127.09, 126.17, 124.28.

5-(5-BROMOTHIOPHEN-2-YL)FURAN-2-CARBALDEHYDE (**7a**).¹⁸ To a stirred solution of **6c** (1 g, 5.62 mmol) in DMF (10 mL) in the dark, was added NBS (1 g, 5.62 mmol) at –23 °C, and the solution was then stirred for 4 h. After this time, the reaction mixture was poured on ice and stirred to room temperature and a light yellow solid precipitated, which was isolated by filtration. Recrystallization from DCM–*n*-hexane gave the pure compound as a light yellow solid (1.37 g, 95%). ^1H NMR (400 MHz, DMSO): δ 9.57 (s, 1H), 7.65 (d, J = 3.7 Hz, 1H), 7.54 (d, J = 3.9 Hz, 1H), 7.37 (d, J = 4.0 Hz, 1H), 7.15 (d, J = 3.7 Hz, 1H); ^{13}C NMR (101 MHz, DMSO): δ 177.46, 152.37, 151.23, 132.42, 132.02, 127.40, 125.38, 113.94, 108.60.

5'-BROMO-[2,2'-BITHIOPHENE]-5-CARBALDEHYDE (**7b**).¹⁸ To a stirred solution of **6d** (1.09 g, 5.62 mmol) in DMF (10 mL) in the dark, was added NBS (1 g, 5.62 mmol) at –23 °C, and the solution was then stirred for 4 h. After this time, the reaction mixture was poured on ice and stirred to room temperature and a light yellow solid precipitated, which was isolated by filtration. Recrystallization from DCM–*n*-hexane gave the pure compound as a light yellow solid (1.47 g, 96%). m.p.: 135–137 °C; ^1H NMR

(400 MHz, CDCl_3): δ 9.87 (s, 1H), 7.66 (d, J = 4.0 Hz, 1H), 7.18 (d, J = 3.9 Hz, 1H), 7.10 (d, J = 3.9 Hz, 1H), 7.04 (d, J = 3.9 Hz, 1H); ^{13}C NMR (101 MHz, CDCl_3): δ 182.51, 145.82, 142.02, 137.44, 137.19, 131.21, 126.21, 124.38, 114.17.

5-(5-BROMOFURAN-2-YL)THIOPHENE-2-CARBALDEHYDE (**8a**).¹⁹ NBS (1.32 g, 7.41 mmol) was gradually added to a stirred solution of **6a** (1.32 g, 7.41 mmol) and BPO (24.2 mg, 0.1 mmol) in toluene (20 mL) over 30 min at –23 °C in air in the dark. After the mixture was stirred for 3.5 h at –23 °C, the solvent was evaporated *in vacuo*. The residue was purified by column chromatography on silica gel (*n*-hexane– CH_2Cl_2 , 4 : 1) to provide the title compound as a light brown solid (1.79 g, 94%). m.p.: 137–139 °C; ^1H NMR (400 MHz, CDCl_3): δ 9.89 (s, 1H), 7.68 (d, J = 4.0 Hz, 1H), 7.32 (d, J = 4.0 Hz, 1H), 6.69 (d, J = 3.5 Hz, 1H), 6.44 (d, J = 3.5 Hz, 1H); ^{13}C NMR (101 MHz, CDCl_3): δ 182.62, 150.05, 141.86, 141.29, 137.04, 123.75, 123.23, 114.14, 110.90.

5'-BROMO-[2,2'-BIFURAN]-5-CARBALDEHYDE (**8b**).¹⁹ NBS (1.32 g, 7.41 mmol) was gradually added to a stirred solution of **6b** (1.20 g, 7.41 mmol) and BPO (24.2 mg, 0.1 mmol) in toluene (20 mL) over 30 min at –23 °C in air in the dark. After the mixture was stirred for 3.5 h at –23 °C, the solvent was evaporated *in vacuo*. The residue was purified by column chromatography on silica gel (*n*-hexane– CH_2Cl_2 , 3 : 1) to provide the title compound as a brown solid (1.61 g, 90%). m.p.: 112–114 °C; ^1H NMR (400 MHz, CDCl_3): δ 9.63 (s, 1H), 7.30 (d, J = 3.8 Hz, 1H), 6.86 (d, J = 3.5 Hz, 1H), 6.75 (d, J = 3.8 Hz, 1H), 6.47 (d, J = 3.5 Hz, 1H); ^{13}C NMR (101 MHz, CDCl_3): δ 177.01, 151.72, 150.11, 146.74, 124.36, 123.17, 114.00, 111.68, 107.83.

General procedure for preparation of compounds **2a**–**d**

NaBH_4 (0.5 equiv) was added, in portions, to **8** or **7** (1 equiv) in 10 mL of CH_3OH – CH_2Cl_2 (1 : 1) in an ice–water bath for 30 min. The solvent was evaporated, then 1 N HCl was poured into the mixture, which was then extracted with CH_2Cl_2 . The combined organic layers were washed with brine, dried (Na_2SO_4), and concentrated *in vacuo* to afford the title compound as a white solid. These compounds were used in the next step as soon as possible due to their instability.

(5-(5-BROMOFURAN-2-YL)THIOPHEN-2-YL)METHANOL (**2a**). The general procedure was used with **8a** (2 g, 7.78 mmol) and NaBH_4 (0.15 g, 3.89 mmol). Afforded the title compound as a white solid (1.98 g, 99%).

(5'-BROMO-[2,2'-BIFURAN]-5-YL)METHANOL (**2b**). The general procedure was used with **8b** (2 g, 8.3 mmol) and NaBH_4 (0.16 g, 4.15 mmol). Afforded the title compound as a white solid (1.96 g, 97%).

(5-(5-BROMOTHIOPHEN-2-YL)FURAN-2-YL)METHANOL (**2c**). The general procedure was used with **7a** (2 g, 7.78 mmol) and NaBH_4 (0.15 g, 3.89 mmol). Afforded the title compound as a white solid (1.98 g, 98%).

(5'-BROMO-[2,2'-BITHIOPHEN]-5-YL)METHANOL (**2d**). The general procedure was used with **7b** (2 g, 7.33 mmol) and NaBH_4

(0.14 g, 3.66 mmol). Afforded the title compound as a white solid (1.96 g, 97%).

General procedure for preparation of compounds 9a–9g, 12a–12b

To a solution of heteroaryl halide 2 (1.0 equiv), boronic acid 3 (1.5 equiv), K_2CO_3 (3.0 equiv), 2-dicyclohexylphosphino-2',4',6'-triisopropylbiphenyl (X-Phos, 0.02 equiv) in CH_3CN-H_2O (1.5 : 1) was added $Pd(OAc)_2$ (0.01 equiv). The flask and its contents were put under reduced pressure then backfilled with argon three times. The mixture was then stirred at room temperature for 24 h under argon atmosphere, the solvent was removed *in vacuo* to afford a crude product, which was purified by flash chromatography (eluting with *n*-hexane-acetone mixtures).

5-(5-(HYDROXYMETHYL)THIOPHEN-2-YL)FURAN-2-YL)THIOPHENE-2-CARBALDEHYDE (**9a**). The general procedure was used with **2a** (0.94 g, 3.64 mmol) and **3a** (5-formylthiophen-2-yl)boronic acid (0.85 g, 5.45 mmol). The product was purified by column chromatography on silica gel (*n*-hexane-acetone, 3 : 1) to provide the title compound as a brown solid (0.68 g, 64%). m.p.: 131–133 °C; 1H NMR (400 MHz, $CDCl_3$): δ 9.87 (s, 1H), 7.69 (d, J = 4.0 Hz, 1H), 7.34 (d, J = 4.0 Hz, 1H), 7.22 (d, J = 3.7 Hz, 1H), 6.97 (d, J = 3.6 Hz, 1H), 6.79 (d, J = 3.6 Hz, 1H), 6.58 (d, J = 3.6 Hz, 1H), 4.85 (s, 2H); ^{13}C NMR (101 MHz, $CDCl_3$): δ 182.50, 150.37, 147.27, 144.30, 142.30, 141.52, 137.18, 132.66, 126.12, 123.50, 122.90, 111.08, 107.73, 60.15; HRMS (ESI): m/z calcd for $C_{14}H_{10}O_3S_2$ [M + H]⁺: 291.0071; found: 291.0153.

5'-(5-(HYDROXYMETHYL)THIOPHEN-2-YL)-[2,2'-BIFURAN]-5-CARBALDEHYDE (**9b**). The general procedure was used with **2a** (0.94 g, 3.64 mmol) and **3b** (5-formylfuran-2-yl)boronic acid (0.76 g, 5.45 mmol). The product was purified by column chromatography on silica gel (*n*-hexane-acetone, 2 : 1) to provide the title compound as a yellow solid (0.54 g, 54%). m.p.: 118–120 °C; 1H NMR (400 MHz, $CDCl_3$): δ 9.59 (s, 1H), 7.31 (d, J = 4.0 Hz, 1H), 7.22 (d, J = 3.6 Hz, 1H), 6.96–6.94 (m, 2H), 6.77 (d, J = 4.0 Hz, 1H), 6.59 (d, J = 3.6 Hz, 1H), 4.84 (s, 2H); ^{13}C NMR (101 MHz, DMSO): δ 176.88, 151.69, 151.14, 150.90, 144.55, 143.79, 132.52, 126.02, 124.27, 123.64, 111.95, 107.57, 107.48, 60.07; HRMS (ESI): m/z calcd for $C_{14}H_{10}O_4S$ [M + H]⁺: 275.0300; found: 275.0386.

5-(5'-(HYDROXYMETHYL)-[2,2'-BITHIOPHEN]-5-YL)FURAN-2-CARBALDEHYDE (**9c**). The general procedure was used with **2d** (1 g, 3.64 mmol) and **3b** (5-formylfuran-2-yl)boronic acid (0.76 g, 5.45 mmol). The product was purified by column chromatography on silica gel (*n*-hexane-acetone, 3 : 1) to provide the title compound as a brown solid (0.62 g, 59%). m.p.: 126–128 °C; 1H NMR (400 MHz, DMSO): δ 9.56 (s, 1H), 7.66 (dd, J = 6.9, 3.9 Hz, 2H), 7.36 (d, J = 3.9 Hz, 1H), 7.29 (d, J = 3.6 Hz, 1H), 7.15 (d, J = 3.8 Hz, 1H), 6.96 (d, J = 3.5 Hz, 1H), 5.61 (s, 1H), 4.64 (s, 2H); ^{13}C NMR (101 MHz, DMSO): δ 177.17, 153.29, 151.06, 147.28, 138.91, 134.09, 128.74, 127.92, 125.67, 125.05, 124.72, 124.59, 108.41, 58.24; HRMS (ESI): m/z calcd for $C_{14}H_{10}O_3S_2$ [M + H]⁺: 291.0071; found: 291.0156.

5''-(HYDROXYMETHYL)-[2,2':5',2''-TERFURAN]-5-CARBALDEHYDE (**9d**). The general procedure was used with **2b** (0.88 g, 3.64 mmol) and

3b (5-formylfuran-2-yl)boronic acid (0.76 g, 5.45 mmol). The product was purified by column chromatography on silica gel (*n*-hexane-acetone, 1 : 1) to provide the title compound as a yellow solid (0.45 g, 48%). m.p.: 178–179 °C; 1H NMR (400 MHz, DMSO): δ 9.59 (s, 1H), 7.68 (d, J = 3.8 Hz, 1H), 7.20 (d, J = 3.7 Hz, 1H), 7.08 (d, J = 3.8 Hz, 1H), 6.89 (d, J = 3.7 Hz, 1H), 6.84 (d, J = 3.3 Hz, 1H), 6.48 (d, J = 3.3 Hz, 1H), 5.38 (t, J = 5.8 Hz, 1H), 4.47 (d, J = 5.8 Hz, 2H); ^{13}C NMR (101 MHz, DMSO): δ 178.00, 156.87, 151.93, 150.09, 147.47, 144.31, 143.60, 125.88, 112.81, 109.77, 108.98, 108.75, 108.31, 56.08; HRMS (ESI): m/z calcd for $C_{14}H_{10}O_5$ [M + H]⁺: 259.0528; found: 259.0602.

5'-(5-(HYDROXYMETHYL)FURAN-2-YL)-[2,2'-BITHIOPHENE]-5-CARBALDEHYDE (**9e**). The general procedure was used with **2c** (0.94 g, 3.64 mmol) and **3a** (5-formylthiophen-2-yl)boronic acid (0.85 g, 5.45 mmol). The product was purified by column chromatography on silica gel (*n*-hexane-acetone, 3 : 1) to provide the title compound as a brown solid (0.67 g, 63%). m.p.: 136–138 °C; 1H NMR (400 MHz, $CDCl_3$): δ 9.86 (s, 1H), 7.68 (d, J = 4.0 Hz, 1H), 7.29 (d, J = 4.0 Hz, 1H), 7.24 (d, J = 4.0 Hz, 1H), 7.20 (d, J = 4.0 Hz, 1H), 6.53 (d, J = 3.2 Hz, 1H), 6.39 (d, J = 3.2 Hz, 1H), 4.67 (s, 2H); ^{13}C NMR (101 MHz, $CDCl_3$): δ 182.36, 154.14, 148.54, 146.86, 141.74, 137.28, 134.96, 134.54, 126.83, 124.08, 123.64, 110.26, 107.18, 57.56; HRMS (ESI): m/z calcd for $C_{14}H_{10}O_3S_2$ [M + H]⁺: 291.0071; found: 291.0152.

5-(5-(HYDROXYMETHYL)FURAN-2-YL)FURAN-2-CARBALDEHYDE (**9f**). The general procedure was used with **2c** (0.94 g, 3.64 mmol) and **3b** (5-formylfuran-2-yl)boronic acid (0.76 g, 5.45 mmol). The product was purified by column chromatography on silica gel (*n*-hexane-acetone, 2 : 1) to provide the title compound as a yellow solid (0.56 g, 56%). m.p.: 133–135 °C; 1H NMR (400 MHz, $CDCl_3$): δ 9.56 (s, 1H), 7.68–7.66 (m, 2H), 7.42 (d, J = 4.0 Hz, 1H), 7.15 (d, J = 3.6 Hz, 1H), 6.87 (d, J = 3.2 Hz, 1H), 6.45 (d, J = 3.2 Hz, 1H), 5.34 (t, J = 5.6 Hz, 1H), 4.44 (d, J = 5.6 Hz, 2H), 4.67 (s, 2H); ^{13}C NMR (101 MHz, $CDCl_3$): δ 177.16, 155.88, 153.37, 151.03, 146.92, 134.60, 128.68, 127.84, 125.74, 123.71, 109.60, 108.44, 107.90, 55.47; HRMS (ESI): m/z calcd for $C_{14}H_{10}O_4S$ [M + H]⁺: 275.0300; found: 275.0372; [M + Na]⁺: 297.0192; found: 297.0241, [M + K]⁺: 313.9935; found: 313.0032.

5''-(HYDROXYMETHYL)-[2,2':5',2''-TERTHIOPHENE]-5-CARBALDEHYDE (**9g**). The general procedure was used with **2d** (5 g, 18.2 mmol) and **3a** (5-formylthiophen-2-yl)boronic acid (4.25 g, 27.25 mmol). The product was purified by column chromatography on silica gel (*n*-hexane-acetone, 3 : 1) to provide the title compound as a brown solid (4.9 g, 88%). m.p.: 166–167 °C; 1H NMR (400 MHz, DMSO): δ 9.89 (s, 1H), 8.00 (d, J = 4.0 Hz, 1H), 7.56 (dd, J = 8.0, 3.9 Hz, 2H), 7.32 (d, J = 3.9 Hz, 1H), 7.27 (d, J = 3.6 Hz, 1H), 6.95 (d, J = 3.6 Hz, 1H), 5.61 (t, J = 5.7 Hz, 1H), 4.64 (d, J = 5.7 Hz, 2H); ^{13}C NMR (101 MHz, DMSO): δ 183.65, 147.23, 144.99, 141.14, 139.07, 138.51, 134.08, 133.27, 127.95, 125.08, 125.03, 124.83, 124.52, 58.24; HRMS (ESI): m/z calcd for $C_{14}H_{10}O_2S_3$ [M + H]⁺: 306.9843; found: 306.9917.

1-(5'-(HYDROXYMETHYL)FURAN-2-YL)-[2,2'-BITHIOPHEN]-5-YL)ETHANONE (**12a**). The general procedure was used with **2c** (0.94 g,

3.64 mmol) and **11** [2-(5-boronothiophen-2-yl)-2-oxoethan-1-ylidium (0.92 g, 5.45 mmol)]. The product was purified by column chromatography on silica gel (*n*-hexane–acetone, 5 : 1) to provide the title compound as a yellow solid (0.71 g, 64%). m.p.: 165–167 °C; ¹H NMR (400 MHz, DMSO): δ 7.91 (d, *J* = 4.0 Hz, 1H), 7.53 (d, *J* = 3.9 Hz, 1H), 7.46 (d, *J* = 4.0 Hz, 1H), 7.36 (d, *J* = 3.9 Hz, 1H), 6.83 (d, *J* = 3.3 Hz, 1H), 6.44 (d, *J* = 3.3 Hz, 1H), 5.35 (t, *J* = 5.8 Hz, 1H), 4.44 (d, *J* = 5.8 Hz, 2H), 2.53 (s, 3H); ¹³C NMR (101 MHz, DMSO): δ 190.83, 156.28, 147.51, 144.50, 142.54, 135.59, 134.21, 127.76, 125.46, 124.28, 110.10, 108.13, 63.27, 26.78; HRMS (ESI): *m/z* calcd for C₁₅H₁₂O₃S₂ [M + H]⁺: 305.0228; found: 305.0306, [M + Na]⁺: 327.012; found: 327.0126, [M + K]⁺: 342.9860; found: 342.9943.

1-(5'-(HYDROXYMETHYL)-[2,2':5',2''-TERTHIOPHEN]-5-YL)ETHANONE (12B). The general procedure was used with **2d** (1 g, 3.64 mmol) and **11** [2-(5-boronothiophen-2-yl)-2-oxoethan-1-ylidium (0.92 g, 5.45 mmol)]. The product was purified by column chromatography on silica gel (*n*-hexane–acetone, 3 : 1) to provide the title compound as a brown solid (0.79 g, 68%). m.p.: 206–207 °C; ¹H NMR (400 MHz, DMSO): δ 7.91 (d, *J* = 4.0 Hz, 1H), 7.50 (d, *J* = 3.9 Hz, 1H), 7.45 (d, *J* = 4.0 Hz, 1H), 7.29 (d, *J* = 3.9 Hz, 1H), 7.24 (d, *J* = 3.6 Hz, 1H), 6.95 (d, *J* = 3.6 Hz, 1H), 5.60 (s, 1H), 4.63 (s, 2H), 2.53 (s, 3H); ¹³C NMR (101 MHz, DMSO): δ 190.81, 147.57, 144.43, 142.55, 138.47, 135.56, 134.75, 134.16, 127.86, 125.58, 125.47, 125.29, 124.89, 58.80, 26.78; HRMS (ESI): *m/z* calcd for C₁₅H₁₂O₂S₃ [M + H]⁺: 320.9999; found: 321.0081.

General procedure for preparation of compounds 1a–1f

All reactions were performed on a 1.0 mmol scale. NaBH₄ (0.5 equiv) was added, in portions, to **9** (1 equiv) in CH₃OH–THF (1 : 1, for a final concentration of 0.1 M) in an ice–water bath for 30 min. The solvent was evaporated *in vacuo*, CH₂Cl₂ was poured into the mixture and, after filtration, the filter cake was washed with CH₂Cl₂ to afford the title compound.

5,5'-(FURAN-2,5-DIYL)BIS(THIOPHENE-5,2-DIYL)DIMETHANOL (RITA: 1A). Yellow solid (0.29 g, 98%). m.p.: 158–160 °C; ¹H NMR (400 MHz, DMSO): δ 7.24 (d, *J* = 3.6 Hz, 2H), 6.95 (d, *J* = 3.6 Hz, 2H), 6.79 (s, 2H), 5.57 (t, *J* = 5.7 Hz, 2H), 4.64 (d, *J* = 5.7 Hz, 4H); ¹³C NMR (101 MHz, DMSO): δ 147.80, 145.94, 131.00, 124.72, 122.48, 107.29, 58.21; HRMS (ESI): *m/z* calcd for C₁₄H₁₂O₃S₂ [M + H]⁺: 293.0228; found: 293.0302.

(5-(5'-(HYDROXYMETHYL)-[2,2'-BIFURAN]-5-YL)THIOPHEN-2-YL)METHANOL (1B). Yellow solid (0.26 g, 95%). m.p.: 142–144 °C; ¹H NMR (400 MHz, CD₃OD): δ 7.19 (d, *J* = 3.6 Hz, 1H), 6.94 (d, *J* = 3.6 Hz, 1H), 6.64 (d, *J* = 3.6 Hz, 1H), 6.62 (d, *J* = 3.6 Hz, 1H), 6.58 (d, *J* = 3.2 Hz, 1H), 6.41 (d, *J* = 3.2 Hz, 1H), 4.73 (s, 2H), 4.56 (s, 2H); ¹³C NMR (101 MHz, CD₃OD): δ 155.90, 150.25, 147.27, 146.87, 145.74, 134.09, 126.78, 123.58, 110.44, 108.28, 107.71, 107.09, 60.06, 57.43; HRMS (ESI): *m/z* calcd for C₁₄H₁₂O₄S [M + H]⁺: 277.04568; found: 277.0535; [M + Na]⁺: 299.0348; found: 299.0354; [M + K]⁺: 315.0088; found: 315.0093.

[2,2':5',2''-TERFURAN]-5,5''-DIYLDIMETHANOL (1c). Gray solid (0.25 g, 95%). m.p.: 186–188 °C. (lit.²⁸ 190–192 °C); ¹H NMR (400 MHz, DMSO): δ 6.74 (s, 2H), 6.68 (d, *J* = 3.3 Hz, 2H), 6.41 (d, *J* = 3.3 Hz, 2H), 5.35 (s, 2H), 4.42 (s, 4H); ¹³C NMR

(101 MHz, DMSO): δ 156.02, 145.29, 144.80, 109.62, 107.74, 107.26, 56.00; HRMS (ESI): *m/z* calcd for C₁₄H₁₂O₅ [M + H]⁺: 261.0685; found: 261.0763; [M + Na]⁺: 283.0577; found: 283.0582; [M + K]⁺: 299.0317; found: 299.0400.

(5-(5'-(HYDROXYMETHYL)-[2,2'-BIFURAN]-5-YL)FURAN-2-YL)METHANOL (1D). Yellow solid (0.29 g, 98%). m.p.: 135–137 °C; ¹H NMR (400 MHz, DMSO): δ 7.27 (d, *J* = 3.8 Hz, 1H), 7.23 (d, *J* = 3.8 Hz, 1H), 7.17 (d, *J* = 3.6 Hz, 1H), 6.92 (d, *J* = 3.6 Hz, 1H), 6.73 (d, *J* = 3.3 Hz, 1H), 6.41 (d, *J* = 3.3 Hz, 1H), 5.56 (s, 1H), 5.31 (s, 1H), 4.62 (s, 2H), 4.42 (s, 2H); ¹³C NMR (101 MHz, DMSO): δ 155.17, 147.29, 146.11, 135.25, 134.74, 131.25, 124.94, 124.23, 123.47, 123.39, 109.36, 106.50, 58.23, 55.50; HRMS (ESI): *m/z* calcd for C₁₄H₁₂O₃S₂ [M – H][–]: 291.0228; found: 291.0115.

(5,5'-(THIOPHENE-2,5-DIYL)BIS(FURAN-5,2-DIYL))DIMETHANOL (1E). Yellow solid (0.27 g, 97%). m.p.: 100–101 °C; ¹H NMR (400 MHz, DMSO): δ 7.30 (s, 2H), 6.73 (d, *J* = 3.3 Hz, 2H), 6.41 (d, *J* = 3.2 Hz, 2H), 5.33 (s, 2H), 4.43 (s, 4H); ¹³C NMR (101 MHz, DMSO): δ 155.74, 147.90, 131.74, 123.86, 109.92, 107.09, 56.07; HRMS (ESI): *m/z* calcd for C₁₄H₁₂O₄S [M + H]⁺: 277.0456; found: 277.0532.

[2,2':5',2''-TERTHIOPHENE]-5,5''-DIYLDIMETHANOL (1F). Yellow solid (0.3 g, 98%). m.p.: 196–198 °C; ¹H NMR (400 MHz, DMSO): δ 7.20 (s, 2H), 7.16 (d, *J* = 3.6 Hz, 2H), 6.92 (d, *J* = 3.5 Hz, 2H), 5.56 (t, *J* = 5.7 Hz, 2H), 4.62 (d, *J* = 5.7 Hz, 4H); ¹³C NMR (101 MHz, DMSO): δ 146.11, 135.29, 134.70, 124.92, 124.34, 123.49, 58.23; HRMS (ESI): *m/z* calcd for C₁₄H₁₂O₂S₃ [M – H][–]: 306.9999; found: 306.9923.

General procedure for preparation of compounds 10a–10d²⁴

All reactions were performed on a 1.0 mmol scale. To a solution of **9** (1 equiv) and Bu₄NBr (0.04 equiv) in CH₂Cl₂–acetone (2 : 1, for a final concentration of 0.1 M) was added TEMPO (0.01 equiv) and Oxone (2.2 equiv). The mixture was then stirred for 1–3 h at room temperature and the solvent was removed under reduced pressure. The product was purified by column chromatography on silica gel (eluting with *n*-hexane–acetone mixtures) to provide the title compound.

5,5'-(FURAN-2,5-DIYL)BIS(THIOPHENE-2-CARBALDEHYDE) (10A). Stirred for 2 h at room temperature, purified by silica gel column chromatography (*n*-hexane–acetone, 6 : 1) to give a brown solid (0.25 g, 86%). m.p.: 186–187 °C; ¹H NMR (400 MHz, DMSO): δ 9.93 (s, 2H), 8.06 (d, *J* = 3.9 Hz, 2H), 7.71 (d, *J* = 3.9 Hz, 2H), 7.35 (s, 2H); ¹³C NMR (101 MHz, DMSO): δ 183.90, 148.34, 141.86, 139.70, 138.77, 125.03, 112.33; HRMS (ESI): *m/z* calcd for C₁₄H₈O₃S₂ [M + H]⁺: 288.9915; found: 288.9993.

5'-(5-FORMYLTHIOPHEN-2-YL)-[2,2'-BIFURAN]-5-CARBALDEHYDE (10B). Stirred for 3 h at room temperature, purified by silica gel column chromatography (*n*-hexane–acetone: 5 : 1) to give a brown solid (0.22 g, 80%). m.p.: 202–204 °C; ¹H NMR (400 MHz, DMSO): δ 9.91 (s, 1H), 9.65 (s, 1H), 7.72 (d, *J* = 4.0 Hz, 1H), 7.43 (d, *J* = 4.0 Hz, 1H), 7.33 (d, *J* = 3.6 Hz, 1H), 7.00 (d, *J* = 3.6 Hz, 1H), 6.87–6.86 (m, 2H); ¹³C NMR (101 MHz, DMSO): δ 182.50, 177.00, 152.14, 150.25, 149.49, 145.48, 142.58, 141.20, 136.89, 124.07, 123.15, 111.94, 110.90, 108.60; HRMS (ESI): *m/z* calcd for C₁₄H₈O₄S [M + H]⁺: 273.0143; found: 273.0223.

5-(5'-FORMYL-[2,2'-BITHIOPHEN]-5-YL)FURAN-2-CARBALDEHYDE (10c). Stirred for 2.5 h at room temperature, purified by silica gel column chromatography (*n*-hexane–acetone: 6 : 1) to give a brown solid (0.23 g, 81%). m.p.: 212–214 °C; ¹H NMR (400 MHz, DMSO): δ 9.91 (s, 1H), 9.59 (s, 1H), 8.04 (d, *J* = 3.9 Hz, 1H), 7.74 (d, *J* = 3.9 Hz, 1H), 7.73–7.63 (m, 3H), 7.25 (d, *J* = 3.8 Hz, 1H); ¹³C NMR (101 MHz, DMSO): δ 183.88, 177.50, 152.65, 151.37, 144.18, 141.94, 139.01, 136.71, 131.74, 128.13, 126.10, 125.47, 109.45; HRMS (ESI): *m/z* calcd for C₁₄H₈O₃S₂ [M + H]⁺: 288.9915; found: 288.9998.

[2,2':5',2"-TERTHIOPHENE]-5,5"-DICARBALDEHYDE (10d). Stirred for 1.5 h at room temperature, purified by silica gel column chromatography (*n*-hexane–acetone: 7 : 1) to give a brown solid (0.26 g, 87%). m.p.: 209–212 °C; ¹H NMR (400 MHz, DMSO): δ 9.91 (s, 2H), 8.03 (d, *J* = 3.9 Hz, 2H), 7.67 (s, 2H), 7.64 (d, *J* = 3.9 Hz, 2H); ¹³C NMR (101 MHz, DMSO): δ = 183.85, 144.16, 141.91, 138.98, 136.35, 128.25, 126.03; HRMS (ESI): *m/z* calcd for C₁₄H₈O₂S₃ [M + H]⁺: 304.9686; found: 304.9766.

General procedure for preparation of compounds 13a–13d

All reactions were performed on a 1.0 mmol scale. Imidazole (2.5 equiv) was added to a solution of **9** (1 equiv) and triisopropylchlorosilane (1.1 equiv) in anhydrous THF (for a final concentration of 0.1 M). The mixture was stirred for 12 h, diluted with 5% NaHCO₃, extracted with EtOAc, washed with brine, dried (Na₂SO₄) and concentrated *in vacuo*. The residue was purified by column chromatography on silica gel (eluting with *n*-hexane–EtOAc mixtures) to provide the title compound as a yellow solid.

5-(5-(5-((TRIISOPROPYLSILYL)OXY)METHYL)THIOPHEN-2-YL)-THIOPHENE-2-CARBALDEHYDE (13a). Purification *via* silica gel column chromatography (*n*-hexane–EtOAc: 70 : 1) to give a yellow solid (0.39 g, 87%). m.p.: 57–59 °C; ¹H NMR (400 MHz, CDCl₃): δ 11.86–11.81 (m, 1H), 9.87 (s, 1H), 7.68 (d, *J* = 4.0 Hz, 1H), 7.34 (d, *J* = 4.0 Hz, 1H), 7.21 (d, *J* = 3.6 Hz, 1H), 6.87 (d, *J* = 3.6 Hz, 1H), 6.78 (d, *J* = 3.6 Hz, 1H), 6.56 (d, *J* = 3.6 Hz, 1H), 4.97 (s, 2H), 1.28–1.06 (m, 22H); ¹³C NMR (101 MHz, CDCl₃): δ 182.43, 150.87, 147.03, 146.44, 142.50, 141.39, 137.15, 131.42, 123.81, 123.40, 122.75, 111.12, 107.31, 61.28, 18.03, 12.09; HRMS (ESI): *m/z* calcd for C₂₃H₃₀O₃S₂Si [M + H]⁺: 447.1406; found: 447.1490.

5"-((TRIISOPROPYLSILYL)OXY)-[2,2':5',2"-TERTHIOPHENE]-5-CARBALDEHYDE (13b). Purification *via* silica gel column chromatography (*n*-hexane–EtOAc: 80 : 1) to give a yellow solid (0.40 g, 86%). m.p.: 61–63 °C; ¹H NMR (400 MHz, CDCl₃): δ 9.85 (s, 1H), 7.66 (d, *J* = 4.0 Hz, 1H), 7.25 (d, *J* = 3.6 Hz, 1H), 7.21 (d, *J* = 4.0 Hz, 1H), 7.09 (d, *J* = 4.0 Hz, 1H), 6.06 (d, *J* = 3.6 Hz, 1H), 6.83 (d, *J* = 4.0 Hz, 1H), 4.95 (s, 2H), 1.11–1.09 (m, 21H); ¹³C NMR (101 MHz, CDCl₃): δ 182.30, 145.99, 146.41, 141.59, 139.73, 137.26, 135.25, 134.18, 126.89, 124.29, 124.03, 123.94, 123.89, 61.29, 18.03, 12.09; HRMS (ESI): *m/z* calcd for C₂₃H₃₀O₂S₃Si [M + H]⁺: 463.1177; found: 463.1268.

5-(5-(5-((TRIISOPROPYLSILYL)OXY)METHYL)FURAN-2-YL)-FURAN-2-CARBALDEHYDE (13c). Purification *via* silica gel column chromatography (*n*-hexane–EtOAc: 70 : 1) to give a yellow solid (0.38 g, 89%). m.p.: 81–83 °C; ¹H NMR (400 MHz, CDCl₃): δ

9.61 (s, 1H), 7.44 (d, *J* = 3.9 Hz, 1H), 7.29 (d, *J* = 3.8 Hz, 1H), 7.20 (d, *J* = 3.9 Hz, 1H), 6.66 (d, *J* = 3.7 Hz, 1H), 6.54 (d, *J* = 3.3 Hz, 1H), 6.34 (d, *J* = 3.3 Hz, 1H), 4.77 (s, 2H), 1.25–1.07 (m, 21H); ¹³C NMR (101 MHz, CDCl₃): δ 176.72, 154.97, 154.66, 151.63, 147.86, 135.91, 129.54, 126.94, 123.48, 123.06, 109.28, 107.51, 107.28, 58.61, 17.99, 12.13. HRMS(ESI): calcd. for C₂₃H₃₀O₄SSi [M + H]⁺: 431.1634; found: 431.1719.

5"-((TRIISOPROPYLSILYL)OXY)-[2,2':5',2"-TERFURAN]-5-CARBALDEHYDE (13d). Purification *via* silica gel column chromatography (*n*-hexane–EtOAc: 70 : 1) to give a yellow solid (0.35 g, 84%). m.p.: 61–63 °C; ¹H NMR (400 MHz, CDCl₃): δ 9.61 (s, 1H), 7.31 (d, *J* = 3.8 Hz, 1H), 6.96 (d, *J* = 3.6 Hz, 1H), 6.77 (d, *J* = 3.8 Hz, 1H), 6.67–6.60 (m, 2H), 6.36 (d, *J* = 3.3 Hz, 1H), 4.78 (s, 2H), 1.09 (dd, *J* = 12.1, 5.4 Hz, 21H); ¹³C NMR (101 MHz, CDCl₃): δ 176.75, 155.15, 151.70, 151.16, 147.99, 144.87, 143.75, 123.39, 111.61, 108.86, 107.49, 107.44, 107.13, 58.58, 17.93, 12.09; HRMS (ESI): *m/z* calcd for C₂₃H₃₀O₅Si [M + H]⁺: 415.1863; found: 415.1939.

General procedure for preparation of compounds 14a–14b

GENERAL PROCEDURES FOR PREPARATION OF OXIMES. All reactions were performed on a 1.0 mmol scale. A mixture of **13** (1 equiv), NH₂OH·HCl (1.5 equiv), AcONa (1.5 equiv) and CH₃OH (for a final concentration of 0.1 M) was refluxed for 2 h and, after filtration, the filter cake was washed with EtOAc three times, then the solvent was removed *in vacuo* to afford the residue, which was purified by column chromatography on silica gel (eluting with *n*-hexane–EtOAc mixtures) to provide the title compound as a yellow solid.

(E)-5"-((TRIISOPROPYLSILYL)OXY)-[2,2':5',2"-TERTHIOPHENE]-5-CARBALDEHYDE OXIME (14a). Purification *via* silica gel column chromatography (*n*-hexane–EtOAc: 30 : 1) to give a yellow solid (0.22 g, 46%). m.p.: 127–131 °C; ¹H NMR (400 MHz, CDCl₃): δ 8.22 (s, 1H), 7.36 (s, 1H), 7.11 (d, *J* = 3.8 Hz, 1H), 7.08 (q, *J* = 3.8 Hz, 2H), 7.05 (d, *J* = 3.8 Hz, 1H), 7.03 (d, *J* = 3.6 Hz, 1H), 6.82 (d, *J* = 3.6 Hz, 1H), 4.94 (s, 2H), 1.23–1.07 (m, 21H); ¹³C NMR (101 MHz, CDCl₃): δ = 145.69, 145.06, 139.25, 137.78, 135.72, 135.15, 133.95, 130.54, 125.26, 124.11, 123.88, 123.50, 123.36, 61.30, 18.04, 12.10; HRMS (ESI): *m/z* calcd for C₂₃H₃₁NO₂S₃Si [M + H]⁺: 478.1286; found: 478.1359.

(Z)-5"-((TRIISOPROPYLSILYL)OXY)-[2,2':5',2"-TERTHIOPHENE]-5-CARBALDEHYDE OXIME (14b). Purification *via* silica gel column chromatography (*n*-hexane–EtOAc: 20 : 1) to give a yellow solid (0.23 g, 48%). m.p.: 122–124 °C; ¹H NMR (400 MHz, CDCl₃): δ 7.69 (s, 1H), 7.28 (d, *J* = 3.9 Hz, 1H), 7.16 (d, *J* = 3.8 Hz, 1H), 7.14 (d, *J* = 3.9 Hz, 1H), 7.06 (d, *J* = 3.8 Hz, 1H), 7.03 (d, *J* = 3.6 Hz, 1H), 6.82 (d, *J* = 3.6 Hz, 1H), 4.94 (s, 2H), 1.23–1.02 (m, 21H); ¹³C NMR (101 MHz, CDCl₃): δ 145.64, 143.07, 141.31, 137.99, 135.77, 135.14, 132.73, 129.27, 125.18, 124.18, 123.90, 123.48, 122.58, 61.30, 18.03, 12.10; HRMS (ESI): *m/z* calcd for C₂₃H₃₁NO₂S₃Si [M + H]⁺: 478.1286; found: 478.1360.

(Z)-5-(5-((TRIISOPROPYLSILYL)OXY)METHYL)THIOPHEN-2-YL)FURAN-2-YL)THIOPHENE-2-CARBALDEHYDE OXIME (14c). Purification *via* silica gel column chromatography (*n*-hexane–EtOAc: 20 : 1) to give a yellow solid (0.21 g, 45%). m.p.: 125–127 °C; ¹H NMR (400 MHz, CDCl₃): δ 7.69 (s, 1H), 7.31 (d, *J* = 3.9 Hz, 1H), 7.27

(s, 1H), 7.18 (d, J = 3.6 Hz, 1H), 6.85 (d, J = 2.8 Hz, 1H), 6.66 (d, J = 3.5 Hz, 1H), 6.53 (d, J = 3.5 Hz, 1H), 4.96 (s, 2H), 1.13 (dd, J = 20.8, 6.1 Hz, 21H); ^{13}C NMR (101 MHz, CDCl_3) δ 149.80, 147.78, 145.55, 141.68, 138.86, 132.66, 131.97, 130.33, 123.83, 122.73, 121.52, 108.94, 107.12, 61.28, 18.03, 12.10. HRMS (ESI): m/z calcd for $\text{C}_{23}\text{H}_{31}\text{NO}_3\text{S}_2\text{Si} [\text{M} + \text{H}]^+$: 462.1515; found: 462.1596.

(5"-(((TRISOPROPYL SILYL)OXY)METHYL)-[2,2':5',2"-TERTHIOPHEN]-5-YL)-METHANOL (15). NaBH_4 (0.5 equiv) was added, in portions, to 9 (1 equiv) in CH_3OH -THF (1:1, for a final concentration of 0.1 M) in an ice-water bath for 30 min. The solvent was evaporated *in vacuo*, then 1 M HCl was poured into the mixture, which was then extracted with CH_2Cl_2 , washed with brine, dried (Na_2SO_4), and concentrated *in vacuo* to afford 15 (0.454 g, 98%) as a yellow solid: m.p.: 32–34 °C; ^1H NMR (400 MHz, CDCl_3): δ 7.03 (s, 2H), 7.02–6.98 (m, 2H), 6.89 (d, J = 3.6 Hz, 1H), 6.81 (d, J = 3.6 Hz, 1H), 4.93 (s, 2H), 4.79 (s, 2H), 1.19–1.07 (m, 21H); ^{13}C NMR (101 MHz, CDCl_3) δ 145.25, 143.03, 137.50, 136.81, 135.94, 135.78, 126.24, 126.19, 124.29, 123.95, 123.85, 123.21, 123.18, 61.27, 60.17, 18.01, 12.06, 12.05; HRMS (ESI): m/z calcd for $\text{C}_{23}\text{H}_{32}\text{O}_2\text{S}_2\text{Si} [\text{M} - \text{H}]^-$: 463.1334; found: 463.1247.

Molecular docking

Docking studies on the representative RITA and compound 1f were performed with the molecular modeling package Discovery Studio.3.1/Libdock protocol (Accelrys Software Inc.). The protein's three dimensional structure from the Brookhaven Protein Data Bank (PDB) was used as a raw model for the docking studies on the X-ray structure of HDM2 in complex with the p53 transactivation domain (PDB code: 1YCR). The general procedure is as follows: (1) ligand and receptor preparation, (2) protocol generation, (3) docking and scoring, (4) analysis of the results. The Discovery Studio.3.1/Standard Dynamics Cascade protocol (Accelrys Software Inc.) was used for molecular dynamics (MD) simulations. The general procedure for MD is as follows: (1) preparing ligand-receptor complex; (2) performing MD simulation, (3) drawing step-potential energy curve and recording the lowest energy conformation, (4) calculating binding energy with the MM/PBSA protocol.

Biological assays

Western blot analysis

The preparation of cell extracts and Western blots were both performed according to standard procedures. Antibodies for immunoblotting were as follows: p53 (Cell Signaling, No. 9282), CDK4 (Santa Cruz, no. sc-260), E2F1 (Santa Cruz, no. sc-193), β -actin (Beyotime Biotechnology, China), horseradish peroxidase-conjugated secondary antibodies (KPL, USA). Treated cells were washed with PBS and lysed in buffer (10 mM HEPES, pH 7.9, 10 mM KCl, 1 mM EDTA, 0.1% NP-40, 1 mM DTT, 1 mM PMSF, 1 mM Pepstatin, 1 mM leupeptin, 0.5 mM

Na_3VO_4). Liquids were kept on ice for 30 min, centrifuged (20 000 rpm, 10 min at 4 °C) and supernatants were used for the assay. Equal amounts of protein extracts were fractionated by SDS-PAGE and transferred to nitrocellulose membranes. After blocking in 5% non-fat dried milk at room temperature for 2 h, the membrane was incubated with primary antibodies and β -actin at 4 °C overnight. The membrane was then incubated with horseradish peroxidase-conjugated goat secondary antibody for 1.5 h at room temperature, and detected with SuperSignal West Pico Chemiluminescent Substrate (Pierce, Thermo). The image was obtained using the Chemi Doc XRS imaging system (Bio-Rad).

Real-time reverse transcription-PCR analysis of microRNA and mRNA expression²⁹

The total RNA from HCT116 was harvested using TRIzol (Invitrogen) according to the manufacturer's protocol. For detecting microRNA expression, reverse reactions were performed using TaqMan MicroRNA Assay kit (Assay no. 4427975; Applied Biosystems, USA). Five micrograms of total RNA were used for the synthesis of first-strand cDNA using the SuperScript III First Strand cDNA Synthesis kit (Invitrogen), following the manufacturer's instructions. Real-time PCR analysis was performed using ABI prism 7500 (Applied Biosystems, USA) with a TaqMan probe provided by the manufacturer. The TaqMan probes used were hsa-mir-34a (Assay no. 4427975; Applied Biosystems, USA) and RNU66 (Assay no. 4427975; Applied Biosystems, USA). The relative amounts of the mRNA and microRNA targeted gene were normalized by the amount of RNU66 transcript.

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