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N-Benzyl-1-heteroaryl-3-(trifluoromethyl)-1*H*-pyrazole-5-carboxamides as inhibitors of co-activator associated arginine methyltransferase 1 (CARM1)

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ABSTRACT

A series of *N*-benzyl-1-heteroaryl-3-(trifluoromethyl)-1*H*-pyrazole-5-carboxamides targeting co-activator associated arginine methyltransferase 1 (CARM1) have been designed and synthesized. The potency of these inhibitors was influenced by the nature of the heteroaryl fragment with the thiophene analogues being superior to thiazole, pyridine, isoindoline and benzofuran based inhibitors.

Mammalian protein arginine methyltransferases (PRMTs) comprise a family of at least nine members with diverse biological functions.[1] They catalyze the transfer of methyl groups from *S*-adenosyl methionine to specific arginine residues, resulting in the formation of either asymmetric (Type I; PRMTs 1–4, 6 and 8) or symmetric (Type II; PRMTs 5, 7 and 9) ω-N_G,N_G-dimethylarginine tails on a wide variety of protein substrates.[2]

PRMT4, also known as co-activator associated arginine methyltransferase 1 (CARM1), methylates proteins with roles encompassing three key levels of gene expression: chromatin remodeling (histone H3 and CBP/p300),[3a–d] RNA processing and stability (PABP, HuR and HuD)[3d–g] and RNA splicing (CA150, SAP49, SmB and U1C).[3h,i] CARM1 was initially identified as a secondary co-activator for the p160 steroid co-activator protein GRIP1 in transcription mediated by nuclear hormone receptors.[4] It has also been shown to be a positive co-regulator for SRC-3, another member of the p160 family[5a] as well as for a variety of other transcription factors such as CBP/p300,[3a,5b] β-catenin[5c] p53,[5d] nuclear factor kappa-B (NF-κB),[5e,f] CIITA,[5g] HTLV-1 Tax,[5h] PPARγ[5i] and c-fos.[5j] The co-activator activity of CARM1 coincides with the arginine methylation of histone H3 and CBP/p300 on several target promoters and mutations of critical residues in the catalytic domain of CARM1 has been shown to compromise transcriptional activation, suggesting that the integrity of the methyltransferase domain CARM1 is important for its co-activator function.[4,5c,g]

Knockout or silencing of CARM1 impedes estrogen-stimulated gene expression, cell cycle progression and growth of breast cancer cells,[3d,6a] and evidence also link CARM1, cyclin E and steroid co-activator overexpression to high-grade breast cancer tumors.[6b] Furthermore, modulation of CARM1 levels affects androgen-dependent transcription and prostate cancer cell growth,[6c] and elevated CARM1 levels correlate with the development of prostate carcinoma as well as the progression of androgen-independent prostate cancer.[6d] These studies support CARM1 as a plausible target for anti-cancer drug development.

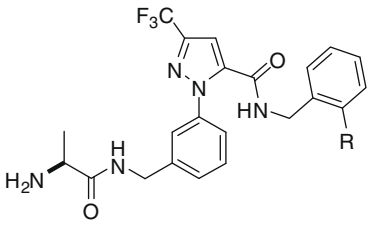

Figure 1. Pyrazole type CARM1 inhibitors. **1a**, R = H, CARM1 IC_{50} = 0.08 μM, Ref. 9b; **1b**, R = OMe.

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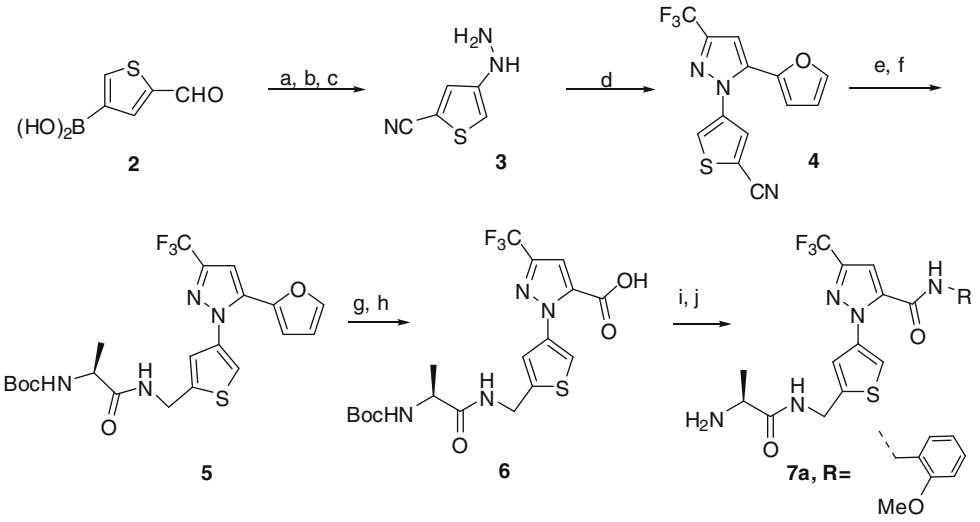


Scheme 1. Reagents and conditions: (a) $N_2(Boc)_2$, $Cu(OAc)_2$, THF, 86%; (b) $NH_2OH{\cdot}HCl$, Pyr, Ac_2O, 80–90 °C, 74%; (c) HCl/dioxane, ^{i}PrOH, 80 °C, 97%; (d) 4,4,4-trifluoro-1-(furan-2-yl)butane-1,3-dione, AcOH, 80 °C, 74%; (e) $BH_3{\cdot}THF$, THF; (f) Boc-Ala-OSu, Et_3N, DCM, 51%; (g) $NaClO_2$, NaH_2PO_4, MeCN, H_2O, 60%; (h) ammonium formate, K_2CO_3, 10% Pd/C, ethanol/reflux, 99%; (i) (2-methoxyphenyl)methanamine, EDC, DMAP, DCM, 51.3%; (j) 1:1 TFA/DCM, 4 h, rt, 99%

Reports describing novel inhibitors of PRMTs originated either from molecular modeling studies or high-throughput screening.[7a–e] However, the majority of these are micro-molar inhibitors that lack selectivity and in some cases they do not possess drug-like properties. Osborne et al. [8] reported the in situ synthesis of a bisubstrate analogue inhibitor of PRMT1 that was over 4-fold more active against PRMT1 compared to CARM1. However, no CARM1 inhibitors exhibiting cellular effects have been described to date. At the time our program began Purandare et al.[9a,9b] described selective CARM1 inhibitors based on the pyrazole scaffold **1** (Fig. 1).

We envisaged the latter inhibitors as a prototype for the design of novel proprietary CARM1 inhibitors. Our initial efforts were focused on the replacement of the central core phenyl ring of **1**[9b] with various heterocycles. To that end, we prepared the 2,4-and 2,5-disubstituted-thiophene analogues shown in Schemes 1 and 2, respectively.[10a] Reaction of 5-formylthiophen-3-ylboronic acid **2** with (E)-di-*tert*-butyl diazene-1,2-dicarboxylate, followed by conversion of the aldehyde to the nitrile using hydroxylamine and acetic anhydride in pyridine, then deprotection gave hydrazine **3**.

Cyclization of hydrazine **3** with 4,4,4-trifluoro-1-(furan-2-yl)-butane-1,3-dione gave pyrazole **4** which was then subjected to borane reduction and the resulting amine (not shown in scheme) was reacted with Boc-Ala-OSu to yield **5**. Oxidation of the furan ring of **5** with $NaClO_2$ produced almost a 1:1 mixture of the desired and the 2-chloro-thiophene analogue of **6** which was readily dehalogenated with ammonium formate and Pd/C to give acid **6**. Coupling of acid **6** with the benzyl amine and deprotection with TFA produced **7a** (Scheme 1).

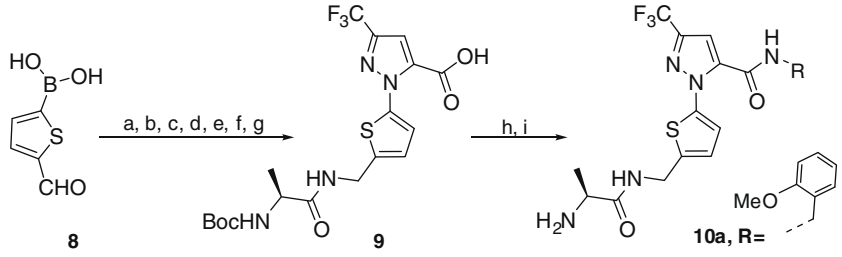
Similarly, the 2,5-disubstituted-thiophene **10a** was prepared starting from 5-formylthiophen-2-ylboronic acid **8**, as depicted in Scheme 2.

The thiazole based analogue was accessible starting from the reported intermediate **11**.[11] Reaction of ester **11** with ammonium hydroxide required heating to produce the carboxamide (not shown in scheme) which was then reduced with diborane and the resulting amine was reacted with Boc-Ala-OSu to give **12**. Intermediate **12** was converted to **13** in three steps using methodologies described above for **7a** and **10a** (Scheme 3).

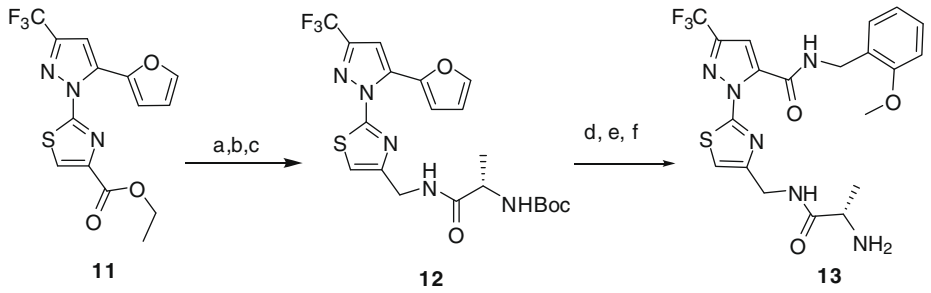
The synthesis of the pyridine scaffold started from 2-chloropyridine **14** which was converted to amine **15** via a sequence of reactions involving displacement of the 2-chloro group of **14** with hydrazine hydrate, cyclization of the resulting hydrazine to the pyrazole, conversion of the alcohol to the azide (not shown in scheme) and reduction of the azide to furnish amine **15**. The remainder of the steps to give **16** is similar to those described for the preparation of **10a** above (Scheme 4).

For the synthesis of the pyridine regioisomer, cyclization of hydrazine **17** with 4,4,4-trifluoro-1-(furan-2-yl)butane-1,3-dione gave a mixture of isomers **18** and **19** in a ratio of 2:1, respectively. The two isomers were separable after the reaction with Boc-Ala-OSu, and the major was taken to the final product **20** (Scheme 5).

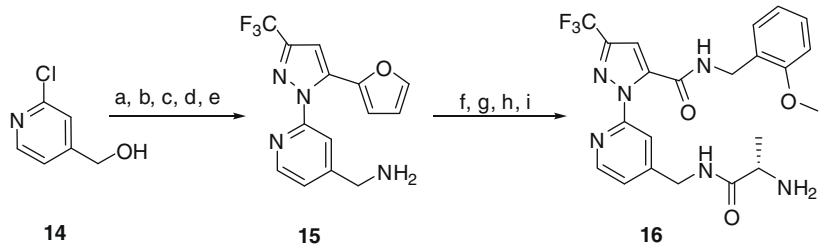
The isoindoline scaffold **24** was synthesized according to Scheme 6, starting from 4-nitroisoindoline-1,3-dione **21**. Reduction of **21** with diborane, protection of the resulting amine with Cbz and reduction of the nitro group followed by diazotization and tin chloride reduction gave the hydrazine, which was con-



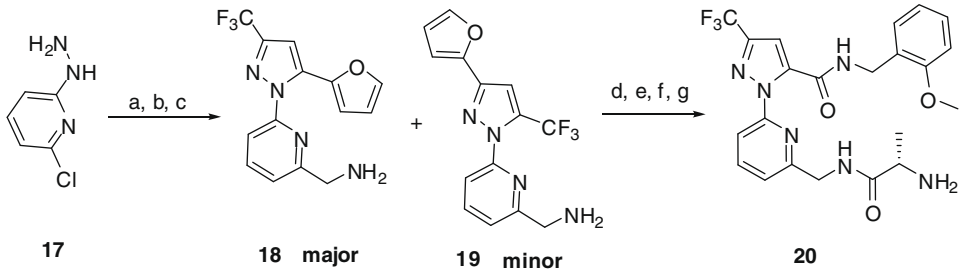
Scheme 2. Reagents and conditions: (a) $N_2(Boc)_2$, $Cu(OAc)_2$, THF, 47%; (b) $NH_2OH{\cdot}HCl$, Pyr, Ac_2O, 80–90 °C, 75%; (c) HCl/dioxane, ^{i}PrOH, 80 °C, 78%; (d) 4,4,4-trifluoro-1-(furan-2-yl)butane-1,3-dione, AcOH, 80 °C, 37%; (e) $BH_3{\cdot}THF$, THF; (f) Boc-Ala-OSu, Et_3N, DCM, 49%; (g) $NaClO_2$, NaH_2PO_4, MeCN, H_2O, 88%; (h) (2-methoxyphenyl)methanamine, $POCl_3$, pyridine, 24%; (i) 1:1 TFA/DCM, 4 h, rt.



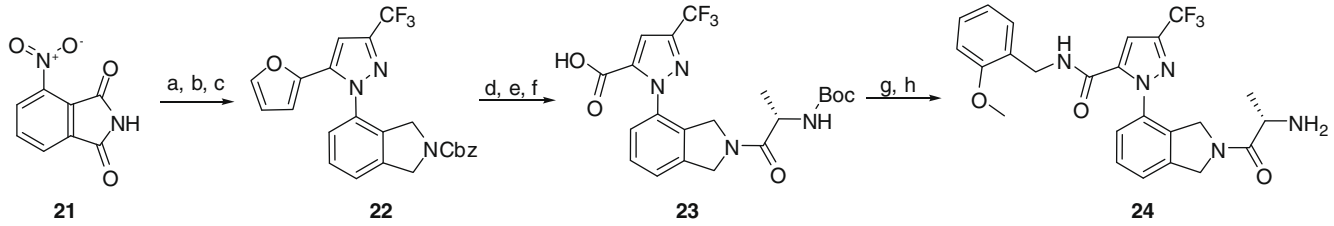
Scheme 3. Reagents and conditions: (a) NH_4OH, MeOH, THF, 16 h at 50 °C; (b) BH_3.THF, THF, 98% over 2 steps; (c) Boc-Ala-OSu, Et_3N, DCM, 21%; (d) $NaClO_2$, NaH_2PO_4, MeCN, H_2O, 90%; (e) (2-methoxyphenyl)methanamine, $POCl_3$, pyridine, 12%; (f) 1:1 TFA/DCM, 4 h, rt, 60%.



Scheme 4. Reagents and conditions: (a) N_2H_2, ^{i}PrOH, 24h, 100 °C THF, 26%; (b) 4,4,4-trifluoro-1-(furan-2-yl)butane-1,3-dione, AcOH, 80 °C, 51%; (c) methanesulfonyl chloride, Et_3N, DCM; (d) NaN_3, DMF, 1 h, 60 °C, 88%; (e) $SnCl_2{\cdot}2H_2O$, MeOH, 2 h, rt., 93%; (f) Boc-Ala-OSu, Et_3N, DCM, 54%; (g) $NaClO_2$, NaH_2PO_4, MeCN, H_2O, 77%; (h) (2-methoxyphenyl)methanamine, $POCl_3$, pyridine, 24%; (i) 1:1 TFA/DCM, 4 h, rt, 80%.



Scheme 5. Reagents and conditions: (a) 4,4,4-trifluoro-1-(furan-2-yl)butane-1,3-dione, AcOH, 80 °C, 2:1 mixture of regioisomers, 90%; (b) $Zn(CN)_2$, $Pd(PPh_3)_4$, DMF, 12%; (c) $BH_3{\cdot}$THF, THF, 99%; (d) Boc-Ala-OSu, Et_3N, DCM, 40%; (e) $RuCl_3$, $NaIO_4$, MeCN, CCl_4, H_2O, quantitative crude yield; (f) (2-methoxyphenyl)methanamine, BOP, Et_3N, DCM, 59%; (g) 1:1 TFA/DCM, 2 h, rt, 56%.

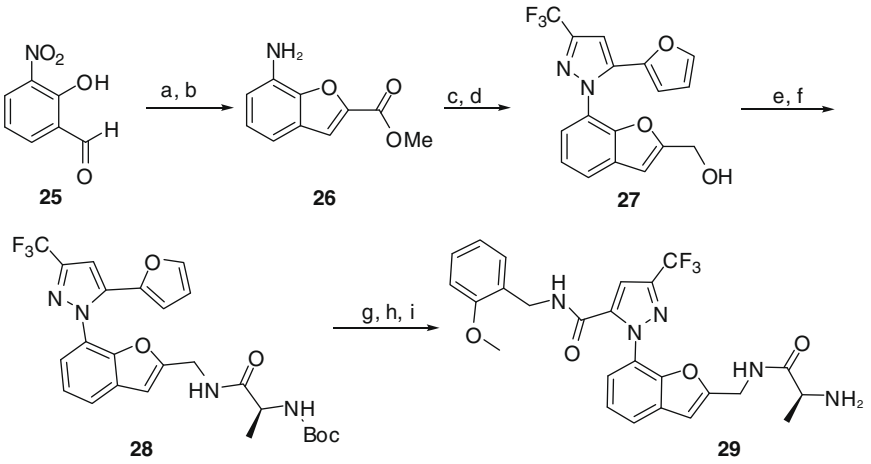


Scheme 6. Reagents and conditions: (a) BH_3.THF, MeOH, crude; (b) Bn-OCOCl, Et_3N, 33%; (c) $SnCl_2{\cdot}2H_2O$, EtOH (d) i—$NaNO_2$; ii—$SnCl_2{\cdot}H_2O$, MeOH, 93%; iii—4,4,4-trifluoro-1-(furan-2-yl)butane-1,3-dione, AcOH, reflux, 16 h, crude.; (e) Boc-Ala-OSu, Et_3N, DCM, 50%; (f) $RuCl_3$, $NaIO_4$, crude; (g) (2-methoxyphenyl)methanamine, BOP, Et_3N, 41%; (h) TFA, DCM, 45%.

verted to pyrazole **22** as described earlier. After removal of the Cbz group of **22**, the produced amine was converted to **24** following the reaction sequence described for **7a**.

The benzofuran scaffold **26** was obtained from **25** by the reaction with dimethyl 2-bromomalonate, followed by catalytic reduction of the nitro group. Diazotization, pyrazole formation and reduction of the ester gave **27** which was transformed to **29** employing procedures described earlier (Scheme 7).

For the construction of the pyrazole ring of compounds **7**, **10**, **13**, **20**, **24** and **29**,[10a] we employed a synthetic methodology similar to that reported for **1a**.[9a,9b] As for thiazole **16** we used a reported procedure for the synthesis of the key intermediate **11**.[11] To confirm that all the compounds above have the desired 3-CF_3 isomer we utilized ^{19}F NMR,[12a] which was able to distinguish between the ^{19}F chemical shift of the 3-CF_3 and the 5-CF_3 pyrazole regioisomers.[12b]



Scheme 7. Reagents and conditions: (a) dimethyl 2-bromomalonate, Bu_4NBr, K_2CO_3, 72%; (b) Pd/C, H_2, MeOH, EtOAc, 99%; (c) i—$NaNO_2$, HCl, 91%; ii—$SnCl_2$, iii—4,4,4-trifluoro-1-(furan-2-yl)butane-1,3-dione, AcOH, 80 °C, 62%; (d) $^iBu_2AlH$, toluene, 86%; (e) i—Ph_3P, imidazole, I_2, DCM, 54%; ii—NaN_3, DMF, 1 h at 60 °C, iii—$SnCl_2{\cdot}2H_2O$, MeOH, 2 h, rt, 82%; (f) Boc-Ala-OSu, Et_3N, DCM, 84%; (g) $NaClO_2$, NaH_2PO_4, MeCN, H_2O; (h) (2-methoxyphenyl)methanamine, BOP, Et_3N, DCM, 10%; (i) 1:1 TFA/DCM, 4 h, rt, 54%.

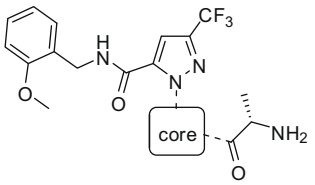
The effects of **7a**, **10a**, **13**, **16**, **20**, **24** and **29** on the activity of CARM1 was measured by means of a histone methyltransferase assay using recombinant CARM1 enzyme, and histone H3 as the substrate[10b] (Table 1). Both the 2,4- and 2,5-disubstituted thiophenes, **7a** and **10a**, were potent inhibitors of CARM1, with IC_{50} of 0.06 and 0.3 μM, respectively. Compound **7a**, was equipotent to **1b**, indicating that the 2,4-substituted thiophene core is a good replacement of the phenyl ring. The thiazole analogue **13** was 36- and 7-fold less active than **7a** and **10a**, respectively, indicating that the nitrogen of the thiazole ring of **13** is less tolerated. Also the pyridine based inhibitors **16** and **20** were 46- and 34-fold less active than **1b**, indicating that the CARM1 enzyme favors a hydrophobic aryl ring over the more polar basic pyridyl moiety. The observed inactivity of isoindoline analogue **24** could be due to the constriction imposed by the less flexible isoindoline ring or the substitution at the benzylic nitrogen. The bicyclic benzofuran core, analogue **29**, was devoid of activity indicating that mono-cyclic rings are preferred. These results confirm that the nature of the core group influences the inhibitory activity of these analogues.

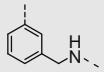
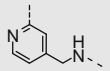
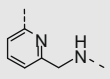
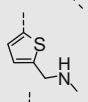
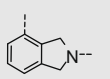
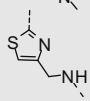
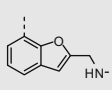
Further SAR exploration centered on the thiophene based analogues. Standard coupling methodologies were employed for the synthesis of amides **7b–g** and **10b–e** using acids **6** and **9** and the appropriate amine.

Table 2 shows the CARM1 inhibitory activity of the thiophene based amides **7b–f** and **10b–e**. For the 2,4-series, scaffold **7**, the substituent on the benzyl group seems to have some effect on the enzymatic activity as compared with the unsubstituted **7c**. The *o*-OMe group is the most favored, with IC_{50} of 0.06 μM, introduction of the *o*-F or *o*-methyl ester substituents (compounds **7b** and **7f**) resulted in a 13-fold loss of activity. A *meta*-OMe group, **7e**, was less tolerated than the ortho analogue, **7a**. Replacement of the benzyl amine with a 2-methylpyridyl, **7g**, had a negative effect reducing the CARM1 activity by 15-fold as compared to **7c**.

Compounds **10a** and **10c** based on the 2,5-thiophene scaffold, displayed similar trends to their counterparts **7a** and **7c**. An *o*-fluoro, *o*-CF_3, or *o*-iso-propyl substituent (**10b**, **10d** and **10e**, respectively) resulted in a marked decrease of activity as compared to **10c**.

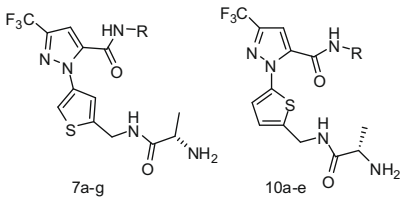
Table 1
Effect of heterocycle core on CARM1 inhibitory activity[a]

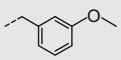
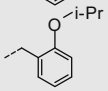
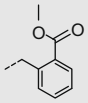


Compound	Core	CARM1 IC_{50} (μM)	Compound	Core	CARM1 IC_{50} (μM)
1b[b]		0.06	**16**		4.20
7a		0.06	**20**		3.05
10a		0.3	**24**		>10
13		2.20	**29**		>10

[a] Values are means of at least two experiments.
[b] **1b**, Figure 1. CARM1 IC_{50} value determined in house.

Table 2
SAR of thiophene scaffolds **7** and **10**[a]



Compound	R	CARM1 IC_{50} (μM)	Compound	R	CARM1 IC_{50} (μM)
7a		0.06	**10a**		0.3
7b		0.79	**10b**		2.31
7c		0.22	**10c**		0.23
7d		0.26	**10d**		4.48
7e		0.37	**10e**		2.92
7f		0.77	**7g**		3.35

[a] Values are means of at least two experiments.

To test the specificity of our inhibitors for CARM1 versus other arginine or lysine methyltransferases, compound **7a** was tested against the PRMT1 arginine and the Set7/9 lysine methyltransferases and was found to be significantly less active against both enzymes (IC_{50} > 100 μM).

To the best of our knowledge, no cellular effects using small molecule CARM1 inhibitors have been reported. We tested compound **7a** for its ability to inhibit in cells the methylation of arginine 26 on histone H3, a target site for CARM1. Treatment of two different cell lines with up to 5 μM of compound **7a** for 48 h did not result in inhibition of histone methylation (data not shown). In the event that the absence of an effect is due to other arginine methyltransferases targeting the same substrate, compound **7a** was also profiled in a variety of functional assays. The assays were targeted towards some of the cancer-related processes in which CARM1 has been shown to be involved, including estrogen-dependent transcription breast cancer cell growth, androgen-dependent signaling and androgen-independent prostate cancer growth. The estrogen-dependent assays entailed measurement of the transactivation of a luciferase reporter driven by three copies of the estrogen response element in response to estradiol,[10c] and a 72 h cell count of estradiol-driven T47D breast ductal carcinoma cells, using the estrogen modulator tamoxifen as the positive control. Androgen-dependent signaling was assessed using a luciferase reporter driven by the MMTV promoter in response to testosterone,[10d] using the androgen antagonist flutamide as the positive control. The MTT cell proliferation assay was used to measure effects on the growth of DU145 prostate carcinoma cells, using the HDAC inhibitor MS-275 as a positive control.[13] As shown in Table 3, compound **7a** had no significant effect on any of the cellular endpoints tested. Compound **1b** was included in our evaluations and also did not show any significant effect in either the methylation or the functional cell-based assays.

Table 3
Cell-based activity of compound **7a**

Compound	Estrogen-dependent transcription T47D-KBluc IC_{50} (μM)	Estrogen-dependent growth T47D IC_{50} (μM)	Androgen-dependent transcription MDA kb2 IC_{50} (μM)	MTT DU145 IC_{50} (μM)
7a	>10	>10	>10	18
1b	>10	>10	>10	18
Tamoxifen	0.1	1	N/A	N/A
Flutamide	N/A	N/A	2	N/A
MS-275	N/A	N/A	N/A	2

N/A, not applicable; NT, not tested.

There are obviously a multitude of potential explanations for these results. One possibility is that CARM1 may be a dispensable component of nuclear receptor-mediated transcription, as it is only a secondary co-activator in this process. Another possibility is that the cellular permeability of the compound may be low and, therefore, may require supra-physiological concentrations of compound to see cellular effects.

In conclusion, we successfully replaced the core phenyl ring with other heterocyclic rings and prepared novel inhibitors based on the 2,4- and 2,5-disubstituted thiophene scaffold which displayed sub-micro-molar activity against CARM1. Thiazole and pyridine based analogues were less potent, while the isoindoline and benzofuran based analogues were devoid of activity. Despite the observed potency against the CARM1 enzyme, our inhibitors did not show measurable cellular activities.

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10. (a) All experimental details can be found in MethylGene patent application, Wahhab, A.; Therrien, E.; Allan, M.; Manku, S. International Patent WO 08/104077 A1, 2008.; (b) The CARM1 enzyme (N-terminal His-tagged, recombinant mouse CARM1, expressed in Sf9 cells) was purchased from Millipore (cat# is 14-575, Lot # is DAM1473541). Histone H3 (Sigma–Aldrich) was used as the substrate, and the methylation was monitored using tritiated *S*-Adenosyl-Methionine (SAM) (Amersham Pharmacia Biotech) as a methyl donor. The reactions were performed at 30 °C for a total of 15 min, using enzyme (CARM1), substrate (histone H3), and co-factor (SAM) in the absence and presence of compound. Assay protocol: 2 μl of the diluted compound was added to a U-Bottom of a PP 96-well plate. CARM1 enzyme was diluted in a Tris–HCl (pH = 9) buffer (to a final enzyme concentration of 0.005 μg/μl) and 8 μl of the cold enzyme solution was immediately added to the compound and allowed to pre-incubate for 10 min at rt. A mixture of SAM (20% [^{3}H]SAM) and histone H3 (10 μl) was then added and incubated for 15 min at 30 °C for a final concentration of 0.0125 μg/μl histone and 2 μM SAM (0.033 mCi/ml). The reaction was stopped by adding 20 μl of SAH for a final concentration of 60 μM and, 10 μl of the quenched reaction is spotted onto P30 Filtermat paper. The Filtermat is washed twice for 15 min with 10% TCA solution and then once for 5 min with 95% ethanol. A wax scintillant is used with the filtermat and the radioactivity was read using a Wallac Microbeta counter (CCPM).; (c) The cell line and methods used for the estrogen-dependent transcriptional assay are described in Wilson, V. S.; Bobseine, K.; Gray, L. E. Jr. *Toxicol. Sci.* **2004**, *81*, 69.; (d) The cell line and methods used for the androgen-dependent transcriptional assay are described in Wilson, V. S.; Bobseine, K.; Lambright, C. R.; Gray, L. E. Jr. *Toxicol. Sci.* **2002**, *66*, 69.
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