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Discovery and structure–activity relationship studies of *N*6-benzoyladenine derivatives as novel BRD4 inhibitors

Tomomi Noguchi-Yachide *, Taki Sakai, Yuichi Hashimoto, Takao Yamaguchi

Institute of Molecular and Cellular, Biosciences, The University of Tokyo, 1-1-1 Yayoi, Bunkyo-ku, Tokyo 113-0032, Japan

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ABSTRACT

Bromodomain and extra-terminal domain (BET) proteins are epigenetic readers that bind to acetylated lysines in histones. Among them, BRD4 is a candidate target molecule of therapeutic agents for diverse diseases, including cancer and inflammatory disease. As a part of our continuing structural development studies of thalidomide to obtain a broad spectrum of biological modifiers based on the 'multi-template' approach, in this work we focused on BRD4-inhibitory activity, and discovered that *N*6-benzoyladenine derivatives exhibit this activity. Structure–activity relationship studies led to *N*6-(2,4,5-trimethoxybenzoyl)adenine (**29**), which exhibits potent BRD4 bromodomain1 inhibitory activity with an IC_{50} value of 0.427 μM. *N*6-Benzoyladenine appears to be a new chemical scaffold for development of BRD4 inhibitors.

1. Introduction

Bromodomains target epigenetic alterations, specifically, ε-acetyl-lysine residues in histones, and serve as regulators of transcriptional activity and chromatin remodeling.[1] The human genome encodes 61 bromodomains in 46 diverse proteins, including histone acetyltransferases (HATs) and chromatin remodeling factors.[2] These bromodomains have been classified into eight major families based on structure and sequence-based identity.[2] The bromodomain and extra-terminal domain (BET) protein family, belonging to Family II, consists of four members (BRD2, BRD3, BRD4 and BRDT), and they control expression of genes related to regulation of various physiological functions, including inflammation, apoptosis, cell proliferation, cell cycle, pancreatic β cell function, and adipogenesis.[3–8] Therefore, BET family members have been receiving increasing attention as candidate therapeutic targets for inflammatory diseases, cancer, diabetes, obesity, cardiovascular diseases and Alzheimer's disease.[9–13] Selective BET inhibitors I-BET762 and RVX-208 (Fig. 1) are already under clinical trial for treatment of nuclear protein in testis (NUT) midline carcinoma (NMC) and atherosclerosis, respectively.[14,15]

Among the BET family, BRD4 binds to acetyl-lysine residues in the tail of histones H3 and H4.[2,16] It induces increased expression of MYC target genes and has been reported to promote transcription of the c-MYC oncogene itself.[10,17–19] In addition, BRD4 recruits positive transcription elongation factor b (P-TEFb), thereby stimulating G_1 gene transcription and promoting cell cycle progression to S phase.[5,6] These activities have potential value for the treatment of cancer. BRD4 inhibitors also have potential as anti-inflammatory agents.[4] BRD4 is a coactivator for transcriptional activation of NF-κB, which mediates inflammation, via binding to acetyl-lysine of RelA, one of the subunits of NF-κB transcriptional complex, and enhances the RNA polymerase II-mediated expression of NF-κB-dependent inflammatory genes, including TNF-α.[5,20]

Thus, there is great interest in discovering new structural scaffolds for BRD4 inhibitors. In this connection, we have proposed the 'multi-template approach' to develop compounds with diverse biological activities by structural development of thalidomide,[21] and we have developed a range of biological modifiers, including anti-angiogenic agents, cyclooxygenase (COX) inhibitors and nuclear receptor ligands.[22–26] Thalidomide is an immunomodulatory and anti-inflammatory agent that was withdrawn from the market in the 1960s due to serious teratogenicity,[21,27] but subsequently re-introduced as a therapeutic agent for multiple myeloma and complications of leprosy, though its action mechanism remains unclear.[27,28] Recent research indicates that thalidomide is a multi-target agent, and might be a lead compound for anti-inflammatory and/or anti-cancer agents. Therefore, we focused here on the bromodomain as a potential new target. Initial screening of thalidomide and its derivatives revealed that *N*6-benzoyladenine showed the desired activity. Subsequent structure–activity studies led us to *N*6-(2,4,5-trimethoxybenzoyl)adenine (**29**) as a potent BRD4 bromodomain1 inhibitor. Our findings indicate that

* Corresponding author. Tel.: +81 3 5841 7875; fax: +81 3 5841 8495.
E-mail address: noguchi@iam.u-tokyo.ac.jp (T. Noguchi-Yachide).

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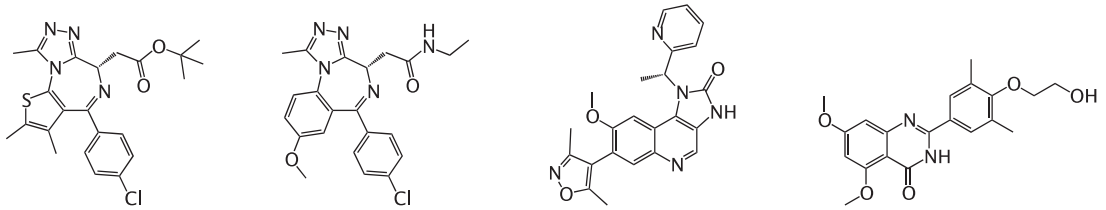


Figure 1. BET bromodomain inhibitors.

*N*6-benzoyladenine is a promising chemical scaffold for developing novel BRD4 inhibitors with smaller molecular weight than previously reported typical BET inhibitors.

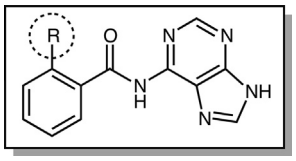
2. Results and discussion

We investigated the inhibitory effects of our compounds on BRD4 bromodomain1 using a commercially available assay kit (BRD4 bromodomain 1 TR-FRET assay kit, Cayman). BRD4-inhibitory activity of test compounds was expressed as IC_{50} values, which were estimated from the sigmoidal dose–response curves using R software and Origin software (Fig. 2 and Tables 1–3; 'N.A.' means that no activity was observed at 100 μM; for compounds that did not achieve 50% inhibition at 300 μM, the % inhibition at 100 μM is shown in parenthesis).

First, we evaluated the BRD4-inhibitory activities of thalidomide (**1**) and three analogs (**2**–**4**) from our chemical library. Compounds **2** and **3** are phthalimide derivatives containing an acetyl-lysine bioisostere instead of the glutarimide moiety of thalidomide (**1**). Compound **4** can be regarded as a ring-opened analog of *N*-heteroaromatic phthalimide structure. Although thalidomide (**1**) and its derivatives (**2**, **3**) were inactive, *N*6-benzoyladenine (**4**) showed moderate activity with an IC_{50} value of 34.2 μM (Table 1). Next, we examined the inhibitory activities of *N*6-benzyladenine (**5**) and its analog *trans*-zeatin (**6**) to investigate the structural requirements for BRD4-inhibitory activity, but both of them were inactive, suggesting that the benzoyl moiety (including the carbonyl group) is essential for the activity. As discussed later, the basicity (or hydrogen bond acceptor ability) of the carbonyl oxygen in the benzoyl moiety, as well as its planar structure, might be important for the activity.

Next, we evaluated the activity of six benzoylated heteroaromatics, **7**–**12**, to investigate the structural requirements of the adenine (heteroaromatic) moiety of *N*6-benzoyladenine (**4**). As shown in Figure 3, none of compounds **7**–**12** achieved 50% inhibition at 100 μM, that is, all of them showed weaker activity than *N*6-benzoyladenine (**4**). The decreased activity of *N*6-benzoyl-9-methyladenine (**7**) might indicate importance of the 9NH group, which could act as a hydrogen bond donor. Of course, various tautomers of *N*6-benzoyladenine (**4**) may exist, so it is possible that any nitrogen atom in the adenine moiety could be both a hydrogen bond donor and a hydrogen bond acceptor. Decreased activity of 4-(benzoylamino)benzimidazole (**8**) suggested that one or both of the 1,3-dinitrogen atoms of the adenine skeleton might be important as a hydrogen bond acceptor(s). Weaker activity of 1-benzoyl-4-(benzoylamino)benzimidazole (**9**) could be attributed to substitution of the 9NH group with the 9-benzoyl group and the absence of nitrogen atoms at the 1- and 3-positions. However, it is

Table 1
BRD4-inhibitory activity of *N*6-(2-substituted benzoyl)adenines



Compound	R	IC_{50} (μM) or (): inhibitory rate at 100 μM
(+)-JQ1	—	0.008
I-BET151	—	0.107
RVX208	—	0.645
N-Benzoyladenine (**4**)	H	34.2
13	NMe_2	5.60
14	OMe	16.0
15	O^nPr	20.0
16	SMe	29.7
17	Me	(40.4%)
18	Br	(39.0%)

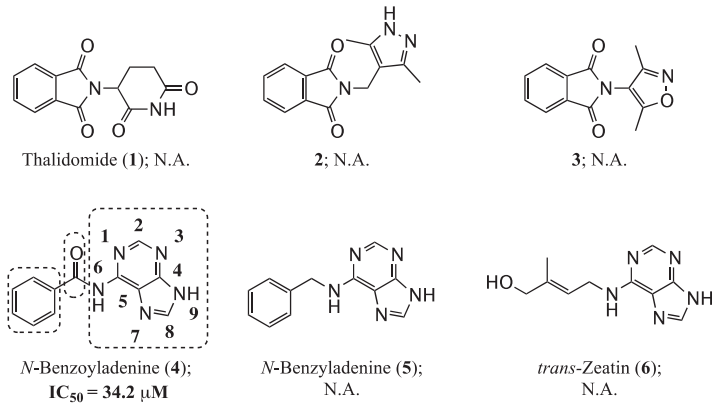
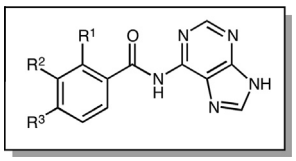


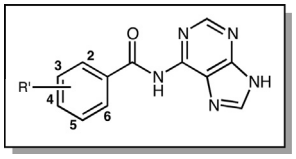
Figure 2. BRD4-inhibitory activity of thalidomide and its analogs.

Table 2
Effect of substitution site on BRD4-inhibitory activity



Compound	R^1	R^2	R^3	IC_{50} (μM) or (): inhibitory rate at 100 μM
(+)-JQ1	—	—	—	0.008
I-BET151	—	—	—	0.107
RVX208	—	—	—	0.645
N-Benzoyladenine (**4**)	H	H	H	34.2
14	OMe	H	H	16.0
18	Br	H	H	(39.0%)
19	H	OMe	H	63.0
20	H	Br	H	(44.5%)
21	H	H	OMe	34.5
22	H	H	Br	(36.7%)

Table 3
Effect of multiple substitution on BRD4-inhibitory activity



Compound	R	IC_{50} (μM) or (): inhibitory rate at 100 μM
(+)-JQ1	—	0.008
I-BET151	—	0.107
RVX208	—	0.645
N-Benzoyladenine (**4**)	H	34.2
23	2,3-OMe	78.6
24	2,4-OMe	(40.8%)
25	2,5-OMe	7.20
26	2,6-OMe	(11.5%)
27	3,4-OMe	7.80
28	3,5-OMe	17.9
29	2,4,5-OMe	0.427

noteworthy that even simple analogs, **10**–**12**, retained weak BRD4-inhibitory activity (Fig. 3).

Next, we investigated the substituent effect at the benzoyl group. The activities of *N*6-(2-substituted benzoyl)adenines (**13**–**18**) are shown in Table 1. The BRD4-inhibitory activity of the compounds decreased in the following order: NMe_2 (**13**) > OMe (**14**) > O^nPr (**15**) > SMe (**16**) > Me (**17**) > Br (**18**). Compounds **13**–**16** showed more potent activity than *N*6-benzoyladenine (**4**). On the other hand, *N*6-(2-methylbenzoyl)adenine (**17**) and *N*6-(2-bromobenzoyl)adenine (**18**) showed weaker activity than *N*6-benzoyladenine (**4**). A possible explanation of these results is that (i) a substituent with hydrogen bond acceptor ability at the 2-position of the benzoyl group, and/or (ii) enhancement of basicity (or hydrogen bond acceptor ability) of the carbonyl oxygen in the benzoyl moiety contribute(s) to the inhibitory activity.

For further structure–activity relationship studies, we investigated the positional effect of substitution on the benzoyl moiety. In accordance with the case of 2-substitution (vide supra, compounds **14** and **18**, Table 1), bromo-substituted compounds (**20**, **22**) were less potent inhibitors than *N*6-benzoyladenine (**4**), while methoxy-substituted compounds (**19**, **21**) showed moderate activities (Table 2). The enhancing effect of a methoxy substituent on the BRD4-inhibitory activity decreased in the order of: *ortho*- (**14**: IC_{50} value of 16.0 μM) > *para*- (**21**: IC_{50} value of 34.5 μM) > *meta*- (**19**: IC_{50} value of 63.0 μM). The observed preference for *ortho*/*para*-methoxy substitution over *meta*-methoxy or bromo substitution implies the importance of the basicity (or hydrogen bond acceptor character) of the carbonyl oxygen in the benzoyl moiety for the activity (vide supra). This interpretation prompted us to investigate the efficacy of multiple substitution (Table 3).

Thus, di- and tri-methoxy derivatives **23**–**29** were prepared and evaluated. Among compounds **23**–**26**, which possess an additional methoxy substituent on *N*6-(2-methoxybenzoyl)adenine (**14**), only *N*6-(2,5-dimethxybenzoyl)adenine (**25**) is more potent than *N*6-(2-methoxybenzoyl)adenine (**14**), having an IC_{50} value of 7.2 μM (Table 3). The other three derivatives were less potent than **14**, and their activity decreased in the following order: **23** (2,3-dimethoxy) > **24** (2,4-dimethoxy) > **26** (2,6-dimethoxy). Thus, introduction of a methoxy group at the *p*-position regarding to the 2-position, that is, the 5-position, is the most effective, implying a role of the basicity (or electron-donating nature) of the oxygen atom in the 2-methoxy group.

Other dimethoxy derivatives with different substitution patterns, that is, compounds **27**, **28**, were also prepared and evaluated. *N*6-(3,4-Dimethoxybenzoyl)adenine (**27**) was more potent than *N*6-(2-methoxybenzoyl)adenine (**14**), having an IC_{50} value of 7.8 μM. *N*6-(3,5-Dimethoxybenzoyl)adenine (**28**) was also moderately active (IC_{50} value of 17.9 μM), having a potency comparable to that of *N*6-(2-methoxybenzoyl)adenine (**14**) and superior to that of *N*6-benzoyladenine (**4**).

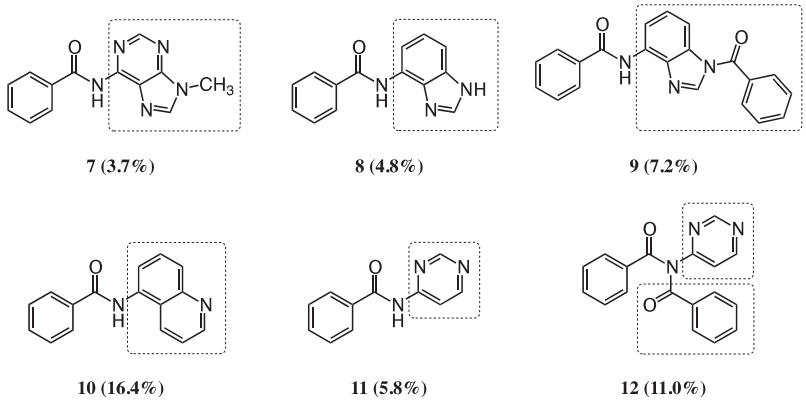


Figure 3. BRD4-inhibitory activity (%) of *N*6-benzoyladenine derivatives at 100 μM.

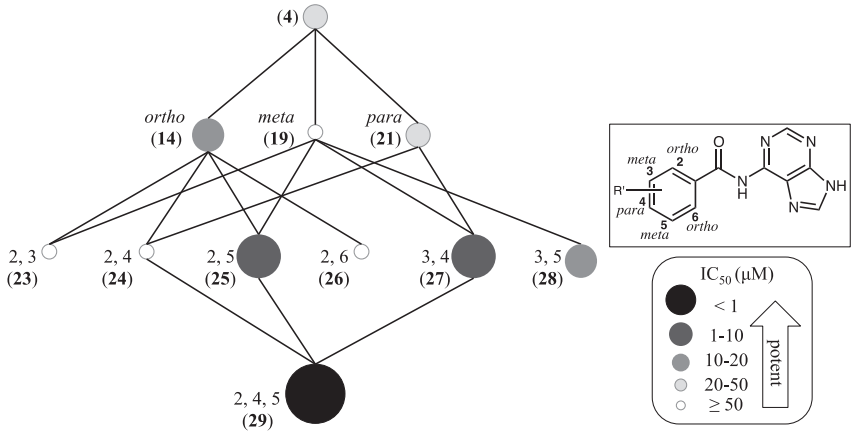


Figure 4. Graphical illustration[30] of the structure–activity relationship for methoxy-substituted derivatives.

The results indicate that 2,5-dimethoxy and 3,4-dimethoxy substitution patterns are preferred. The latter substitution pattern can be considered as the same as 4,5-dimethoxy substitution, because the 3- and 5-positions are both *meta* to the *N*-carbonyladenyl moiety. Therefore, we next focused on the 2,4,5-trimethoxy derivative (Fig. 4). The structure–activity relationships for methoxy-substituted derivatives (**14**, **19**, **21**, **23**–**29**) are graphically illustrated in Figure 4, based on the method similar with that of reported by Zhang et al.[30] In Figure 4, nodes represent substitution sites or site combinations that are scaled in size/color density according to the potency of BRD4-inhibitory activity. As expected, *N*6-(2,4,5-trimethoxybenzoyl)adenine (**29**) was the most potent inhibitor (IC_{50} value of 0.427 μM) among the *N*6-benzoyladenine derivatives prepared in this paper (**7**–**29**), being 80-fold more potent than *N*6-benzoyladenine (**4**). Its potency is comparable to those of the reported inhibitors I-BET151 and RVX208 (Fig. 1).[10,29]

3. Conclusions

Our continuing investigations of thalidomide derivatives based on the 'multi-template approach' led to the discovery that *N*6-benzoyladenine derivatives exhibit BRD4-inhibitory activity. Structure–activity relationship studies led to the identification of *N*6-(2,4,5-trimethoxybenzoyl)adenine (**29**) as a potent small-molecular BRD4 inhibitor. *N*6-Benzoyladenine appears to be a new chemical scaffold for development of BRD4 inhibitors.

4. Experimental section

4.1. General chemistry

All chemical reagents and solvents were purchased from Sigma–Aldrich Co., LLC, Kanto Chemical Co., Inc., Tokyo Chemical Industry Co., Ltd and Wako Pure Chemical Industries, Ltd, and used without further purification. Moisture-sensitive reactions were performed under an atmosphere of argon, unless otherwise noted, and monitored by thin-layer chromatography (TLC, Merck silica gel 60 F_{254} plates). Bands were visualized using UV light or by application of appropriate reagents followed by heating. Flash chromatography was carried out with silica gel (Silica gel 60N, 40–50 μm particle size) purchased from Kanto Chemical Co., Inc. Melting points (Mp) were determined by using a MP-J3 melting point apparatus (Yanaco). NMR spectra were recorded on a JEOL JNM-GX500 (500 MHz) spectrometer, operating at 500 MHz for ^{1}H NMR and at 125 MHz for ^{13}C NMR. Proton and carbon chemical shifts are expressed in δ values (ppm) relative to internal tetramethylsilane (0.00 ppm) or residual $CHCl_3$ (7.26 ppm) for ^{1}H NMR, and internal tetramethylsilane (0.00 ppm) or $CDCl_3$ (77.16 ppm) for ^{13}C NMR. Data are reported as follows: chemical shift, multiplicity (s, singlet; d, doublet; t, triplet; q, quartet; m, multiplet; br, broad), coupling constants (Hz), integration. Fast atom bombardment mass (FAB-MS) spectra were recorded on a JEOL JMA-HX110 mass spectrometer with *m*-nitrobenzyl alcohol as the matrix. High-resolution mass spectrum was recorded using a Bruker micrOTOF II mass spectrometer.

4.1.1. *N*6-Benzoyl-9-methyladenine (7)

Sodium hydride (60 wt % in oil, 24 mg, 0.75 mmol) was added to a solution of *N*6-benzoyladenine (150 mg, 0.63 mmol) in anhydrous DMF (2.0 mL), and the mixture was stirred for 5 min at room temperature. Iodomethane (47 μL, 0.75 mmol) was added, and the resulting suspension was stirred for 14 h at room temperature. Brine was added, and the resulting mixture was extracted with AcOEt. The combined organic layer was dried over Na_2SO_4 and concentrated under reduced pressure. The residue was purified by silica gel column chromatography (MeOH/$CHCl_3$, 1:40) to afford the product **7** (84 mg, 53%) as a white solid. The same compound was also alternatively synthesized by benzoylation of 9-methyladenine.[31] Mp 78.5–80.5 °C; ^{1}H NMR (500 MHz, $CDCl_3$) δ 3.84 (s, 3H), 7.42 (dd, J = 7.8, 7.8 Hz, 2H), 7.51 (tt, J = 1.3, 7.5 Hz, 1H), 7.93 (s, 1H), 7.97 (d, J = 7.5 Hz, 2H), 8.71 (s, 1H), 9.52 (br s, 1H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 30.0, 122.9, 128.0, 128.7, 132.7, 133.7, 143.6, 149.5, 152.4, 152.6, 165.0; FAB-MS m/z 254 $(M+H)^+$.

4.1.2. 4-(Benzoylamino)benzimidazole (8) and 1-Benzoyl-4-(benzoylamino)benzimidazole (9)

Benzoyl chloride (87 μL, 0.75 mmol) was added to a solution of 4-amino-1,3-benzodiazole[32] (100 mg, 0.75 mmol) in anhydrous pyridine (1.0 mL) at 0 °C, and the mixture was stirred for 12 h at room temperature. After removal of the solvent, the residue was purified by silica gel column chromatography (MeOH/$CHCl_3$, 1:20 to 1:10) to afford the products **8** (28 mg, 16%) and **9** (46 mg, 18%), each as a white solid. Compound **8**: mp 183–185 °C; ^{1}H NMR (500 MHz, $CDCl_3$/CD_3OD, 99:1) δ 7.20 (dd, J = 8.0, 8.0 Hz, 1H), 7.26 (d, J = 8.0 Hz, 1H), 7.43 (dd, J = 7.5, 7.5 Hz, 2H), 7.50 (t, J = 7.0 Hz, 1H), 7.88 (s, 1H), 7.98 (d, J = 7.5 Hz, 2H), 8.03 (d, J = 7.5 Hz, 1H), 9.51 (br s, 1H); ^{13}C NMR (125 MHz, $CDCl_3$/CD_3OD, 99:1) δ 109.5, 113.1, 123.6, 127.4, 127.9, 128.7, 131.5, 132.0, 134.5, 135.7, 139.9, 166.8; FAB-MS m/z 238 $(M+H)^+$. Compound **9**: mp 152–154 °C; ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.46 (dd, J = 8.0, 8.0 Hz, 1H), 7.51 (dd, J = 7.5, 7.5 Hz, 2H), 7.55–7.62 (m, 3H), 7.70 (t, J = 7.5 Hz, 1H), 7.79–7.83 (m, 3H), 8.00 (d, J = 8.5 Hz, 2H), 8.16 (s, 1H), 8.54 (d, J = 8.0 Hz, 1H), 9.13 (br s, 1H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 110.7, 114.6, 127.0, 127.3, 128.9,

129.2, 129.7, 130.4, 132.2, 132.2, 132.8, 133.5, 134.4, 134.7, 141.8, 165.7, 167.1; FAB-MS *m*/*z* 342 $(M+H)^+$.

4.1.3. 4-(Benzoylamino)pyrimidine (11) and 4-(Dibenzoylamino)pyrimidine (12)

Compound **11** was prepared by a modification of a literature method.[33] Triethylamine (220 μL, 1.6 mmol) and benzoyl chloride (122 μL, 1.1 mmol) were added to a solution of 4-aminopyrimidine (50 mg, 0.53 mmol) in anhydrous DMF (0.5 mL), and the mixture was stirred for 6 h at room temperature. The precipitate was collected by filtration and washed with hexane, and the filtrate was concentrated under reduced pressure. The residue was purified by silica gel column chromatography (AcOEt/hexane, 1:3 to 1:2) to afford the products **11** (54 mg, 51%) and **12** (25 mg, 16%), each as a white solid. Compound **11**: mp 135.5–137 °C; ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.47 (dd, *J* = 8.8, 8.8 Hz, 2H), 7.57 (t, *J* = 7.5 Hz, 1H), 7.89 (d, *J* = 8.0 Hz, 2H), 8.32 (dd, *J* = 1.5, 6.0 Hz, 1H), 8.61 (d, *J* = 5.5 Hz, 1H), 8.66 (d, *J* = 1.0 Hz, 1H), 9.40 (br s, 1H); ^{13}C NMR (125 MHz, $CDCl_3$) δ110.6, 127.6, 129.0, 133.0, 133.5, 157.6, 158.4, 158.6, 166.8; FAB-MS *m*/*z* 200 $(M+H)^+$. Compound **12**: mp 132.5–133.5 °C; ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.37 (dd, *J* = 7.8, 7.8 Hz, 4H), 7.42 (dd, *J* = 1.5, 5.5 Hz, 1H), 7.50 (tt, *J* = 1.3, 7.5 Hz, 2H), 7.75 (dd, *J* = 1.4, 8.3 Hz, 4H), 8.73 (d, *J* = 6.0 Hz, 1H), 8.90 (d, *J* = 1.0 Hz, 1H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 116.9, 129.0, 129.4, 133.3, 134.1, 158.2, 158.9, 160.7, 172.4; FAB-MS *m*/*z* 304 $(M+H)^+$.

4.1.4. General synthetic procedure (Procedure A) for *N*6-benzoyladenine derivatives 14, 18–22 and 26

N,*N*-Diisopropylethylamine (5.0 equiv) or triethylamine (5.0 equiv) was added to a suspension of adenine (1.0 equiv) in anhydrous DMF (0.4 M). To this mixture was added the appropriate benzoyl chloride (1.2 equiv), and the resulting mixture was stirred at 110–130 °C. After completion of the reaction, the mixture was diluted with water and extracted with AcOEt. The combined organic layer was washed with brine and dried over Na_2SO_4. After removal of the solvent, the residue was purified by recrystallization ($MeOH/CH_2Cl_2$) or by silica gel column chromatography ($MeOH/CHCl_3$) to afford the product. Compound **20** was purified as follows: after completion of the reaction, the mixture was diluted with water, and the precipitate was collected by filtration and washed with water and AcOEt to afford the product.

4.1.4.1. *N*6-(2-Methoxybenzoyl)adenine (14). Pale yellow solid (22%): mp 235–237 °C; ^{1}H NMR (500 MHz, DMSO-d_6) δ 4.08 (s, 3H), 7.23 (dd, *J* = 7.5, 7.5 Hz, 1H), 7.36 (d, *J* = 8.0 Hz, 1H), 7.69–7.72 (m, 1H), 8.01 (dd, *J* = 1.7, 8.0 Hz, 1H), 8.55 (s, 1H), 8.74 (s, 1H), 11.07 (br s, 1H), 12.45 (br s, 1H); FAB-MS *m*/*z* 270 $(M+H)^+$.

4.1.4.2. *N*6-(2-Bromobenzoyl)adenine (18). Brown solid (42%): mp 223–225 °C; ^{1}H NMR (500 MHz, $CDCl_3/CD_3OD$, 100:1) δ 7.43–7.51 (m, 2H), 7.69 (d, *J* = 7.4 Hz, 1H), 7.73 (d, *J* = 7.4 Hz, 1H), 8.39 (s, 1H), 8.75 (s, 1H); FAB-MS *m*/*z* 318 $(M+H)^+$.

4.1.4.3. *N*6-(3-Methoxybenzoyl)adenine (19). Brown solid (45%): mp 237–240 °C; ^{1}H NMR (500 MHz, DMSO-d_6) δ 3.85 (s, 3H), 7.22 (dd, *J* = 2.3, 8.0 Hz, 1H), 7.47 (dd, *J* = 7.7, 7.7 Hz, 1H), 7.68–7.70 (m, 2H), 8.48 (s, 1H), 8.72 (s, 1H); FAB-MS *m*/*z* 270 $(M+H)^+$.

4.1.4.4. *N*6-(3-Bromobenzoyl)adenine (20). White solid (44%): mp >300 °C (reached maximum measurement limit); ^{1}H NMR (500 MHz, DMSO-d_6) δ 7.53 (dd, *J* = 8.0, 8.0 Hz, 1H), 7.86 (d, *J* = 8.0 Hz, 1H), 8.08 (d, *J* = 7.4 Hz, 1H), 8.51 (s, 1H), 8.28 (s, 1H), 8.73 (s, 1H), 11.66 (br s, 1H), 12.38 (br s, 1H); FAB-MS *m*/*z* 318 $(M+H)^+$.

4.1.4.5. *N*6-(4-Methoxybenzoyl)adenine (21). Pale yellow solid (42%): mp 233–236 °C; ^{1}H NMR (500 MHz, $CDCl_3/CD_3OD$, 100:1) δ 3.93 (s, 3H), 7.06 (d, *J* = 8.6 Hz, 2H), 7.33 (s, 1H), 8.06 (d, *J* = 8.6 Hz, 2H), 8.38 (s, 1H), 8.74 (s, 1H); FAB-MS *m*/*z* 270 $(M+H)^+$.

4.1.4.6. *N*6-(4-Bromobenzoyl)adenine (22). Brown solid (42%): mp >300 °C (reached maximum measurement limit); ^{1}H NMR (500 MHz, DMSO-d_6) δ 7.78 (d, *J* = 8.3 Hz, 2H), 8.03 (d, *J* = 8.3 Hz, 2H), 8.49 (s, 1H), 8.72 (s, 1H); FAB-MS *m*/*z* 318 $(M+H)^+$.

4.1.4.7. *N*6-(2,6-Dimethoxybenzoyl)adenine (26). White solid (1%): mp 204–206 °C; ^{1}H NMR (500 MHz, $CDCl_3$) δ 3.81 (s, 6H), 6.69 (d, *J* = 8.6 Hz, 2H), 7.51 (t, *J* = 8.6 Hz, 1H), 7.99 (s, 1H), 8.54 (s, 1H); FAB-MS *m*/*z* 300 $(M+H)^+$.

4.1.4.8. 2-(Methylthio)benzoic acid. This compound was prepared by means of a literature method.[34] Thiosalicylic acid (300 mg, 2.0 mmol) and K_2CO_3 (809 mg, 5.9 mmol) were dissolved in anhydrous acetone (20 mL). Iodomethane (134 μL, 2.2 mmol) was slowly added to the solution, and the mixture was stirred for 20 h. The solvent was removed and the residue was taken up in water and acidified to pH ~1 with 2 N HCl. The precipitated white solid was washed with H_2O and dried to afford the product (260 mg, 79%) as a white solid. ^{1}H NMR (500 MHz, DMSO-d_6) δ 2.38 (s, 3H), 7.19 (dd, *J* = 7.4, 7.4 Hz, 1H), 7.34 (d, *J* = 8.0 Hz, 1H), 7.52–7.55 (m, 1H), 7.89 (dd, *J* = 1.4, 7.7 Hz, 1H); FAB-MS *m*/*z* 168 $(M)^+$.

4.1.5. General synthetic procedure (Procedure B) for *N*6-benzoyladenine derivatives 13, 16, 23–25, and 28

The appropriate benzoic acid (1.2 equiv), 1-[3-(dimethylamino)propyl]-3-ethylcarbodiimide hydrochloride (1.2 equiv) and 4-(dimethylamino)pyridine (1.2 equiv) were added to a suspension of adenine (1.0 equiv) in anhydrous DMF (0.6 M), and the mixture was stirred for 10 h at 130 °C. The resulting mixture was diluted with water and extracted with AcOEt. The combined organic layer was washed with brine and dried over Na_2SO_4. After removal of the solvent, the residue was purified by silica gel column chromatography ($MeOH/CHCl_3$) to afford the product.

4.1.5.1. *N*6-[2-(Dimethylamino)benzoyl]adenine (13). White solid (12%): mp 220–222 °C; ^{1}H NMR (500 MHz, DMSO-$\boldsymbol{d_6}$) $\boldsymbol{\delta}$ 2.82 (s, 6H), 7.29 (dd, ***J*** = 7.4, 7.4 Hz, 1H), 7.49 (d, ***J*** = 8.0 Hz, 1H), 7.60–7.63 (m, 1H), 8.04 (dd, ***J*** = 1.7, 7.4 Hz, 1H), 8.46 (s, 1H), 8.66 (s, 1H); FAB-MS ***m*/*z*** 283 $(M+H)^+$.

4.1.5.2. *N*6-[2-(Methylthio)benzoyl]adenine (16). Pale yellow solid (12%): mp 127–130 °C; ^{1}H NMR (500 MHz, $CDCl_3$) δ 2.56 (s, 3H), 7.32–7.35 (m, 1H), 7.45 (d, *J* = 7.4 Hz, 1H), 7.56 (ddd, *J* = 1.5, 7.7, 7.7 Hz, 1H), 7.82 (dd, *J* = 1.5, 7.7 Hz, 1H), 8.36 (s, 1H), 8.73 (s, 1H), 9.62 (br s, 1H), 11.62 (br s, 1H); FAB-MS *m*/*z* 286 $(M+H)^+$.

4.1.5.3. *N*6-(2,3-Dimethoxybenzoyl)adenine (23). White solid (25%): mp 208–211 °C; ^{1}H NMR (500 MHz, DMSO-d_6) δ 3.88 (s, 3H), 3.90 (s, 3H), 7.25 (dd, *J* = 8.0, 8.0 Hz, 1H), 7.32 (dd, *J* = 1.7, 8.0 Hz, 1H), 7.39 (d, *J* = 6.9 Hz, 1H), 8.48 (s, 1H), 8.68 (s, 1H), 11.24 (br s, 1H), 12.35 (br s, 1H); FAB-MS *m*/*z* 300 $(M+H)^+$.

4.1.5.4. *N*6-(2,4-Dimethoxybenzoyl)adenine (24). White solid (1.5%): mp 267–270 °C; ^{1}H NMR (500 MHz, $CDCl_3$) δ 3.92 (s, 3H), 4.14 (s, 3H), 6.60 (d, *J* = 2.3 Hz, 1H), 6.71 (dd, *J* = 2.3, 8.6 Hz, 1H), 8.26 (d, *J* = 8.6 Hz, 1H), 8.30 (d, *J* = 1.1 Hz, 1H), 8.80 (s, 1H), 10.75 (br s, 1H), 11.81 (br s, 1H); FAB-MS *m*/*z* 300 $(M+H)^+$.

4.1.5.5. *N*6-(2,5-Dimethoxybenzoyl)adenine (25). White solid (21%): mp 213–216 °C; ^{1}H NMR (500 MHz, $CDCl_3$) δ 3.88 (s,

3H), 4.13 (s, 3H), 7.06 (d, J = 8.8 Hz, 1H), 7.18 (dd, J = 3.1, 8.8 Hz, 1H), 7.81 (d, J = 3.1 Hz, 1H), 8.32 (d, J = 1.1 Hz, 1H), 8.82 (s, 1H), 11.01 (br s, 1H), 11.76 (br s, 1H); FAB-MS m/z 300 $(M+H)^+$.

4.1.5.6. *N*6-(3,5-Dimethoxybenzoyl)adenine (28). White solid (43%): mp 242–245 °C; ^{1}H NMR (500 MHz, $CDCl_3$) δ 3.89 (s, 6H), 6.73 (t, J = 2.3 Hz, 1H), 7.09 (s, 1H), 7.10 (s, 1H), 8.36 (s, 1H), 8.81 (s, 1H), 9.01 (br s, 1H), 11.62 (br s, 1H); FAB-MS m/z 300 $(M+H)^+$.

4.1.6. General synthetic procedure (Procedure C) for *N*6-benzoyladenine derivatives 15, 17, 27, and 29

The appropriate benzoic acid (1.0 equiv) and 1-[3-(dimethylamino)propyl]-3-ethylcarbodiimide hydrochloride (1.2 equiv) were added to a suspension of adenine (1.0 equiv) in anhydrous DMF (0.6 M), and the mixture was stirred at 100–130 °C. After completion of the reaction, the solvent was removed under reduced pressure. The residue was then purified by silica gel column chromatography ($MeOH/CHCl_3$) and by washing with CH_2Cl_2/hexane (1:1 or 1:0) to afford the product.

4.1.6.1. *N*6-[2-(*n*-Propoxy)benzoyl]adenine (15). White solid (20%): mp 266.5–268 °C; ^{1}H NMR (500 MHz, $CDCl_3/CD_3OD$, 10:1) δ 1.12 (t, J = 7.0 Hz, 3H), 2.01 (tq, J = 7.1, 7.1 Hz, 2H), 4.18 (t, J = 6.5 Hz, 2H), 7.03 (d, J = 8.5 Hz, 1H), 7.08 (dd, J = 7.3, 7.3 Hz, 1H), 7.52 (ddd, J = 1.5, 7.8, 7.8 Hz, 1H), 8.18 (dd, J = 2.0, 8.0 Hz, 1H), 8.30 (s, 1H), 8.66 (s, 1H), 11.08 (br s, 1H), 12.00 (br s, 1H); ^{13}C NMR (125 MHz, $CDCl_3/CD_3OD$, 10:1) δ 10.5, 22.3, 71.4, 112.8, 113.1, 119.1, 121.5, 132.4, 135.2, 144.1, 144.2, 152.2, 157.7, 161.4, 164.4; FAB-MS m/z 298 $(M+H)^+$.

4.1.6.2. *N*6-(2-Methylbenzoyl)adenine (17). White solid (15%): mp 197–197.5 °C; ^{1}H NMR (500 MHz, $CDCl_3/CD_3OD$, 99:1) δ 2.54 (s, 3H), 7.26–7.33 (m, 2H), 7.44 (dd, J = 7.5, 7.5 Hz, 1H), 7.61 (dd, J = 1.0, 7.5 Hz, 1H), 8.34 (s, 1H), 8.48 (s, 1H), 9.93 (br s, 1H), 11.82 (br s, 1H); ^{13}C NMR (125 MHz, $CDCl_3/CD_3OD$, 99:1) δ 20.2, 113.0, 126.3, 127.4, 131.9, 133.7, 137.4, 143.8, 144.4, 152.2, 162.2, 169.5; FAB-MS m/z 254 $(M+H)^+$.

4.1.6.3. *N*6-(3,4-Dimethoxybenzoyl)adenine (27). White solid (25%): mp 223.5–224.5 °C; ^{1}H NMR (500 MHz, $CDCl_3$) δ 3.98 (s, 3H), 3.98 (s, 3H), 6.98 (d, J = 8.5 Hz, 1H), 7.55 (d, J = 2.5 Hz, 1H), 7.62 (dd, J = 2.5, 8.5 Hz, 1H), 8.36 (s, 1H), 8.76 (s, 1H), 9.15 (br s, 1H), 11.71 (br s, 1H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 56.3, 56.4, 110.8, 110.8, 113.0, 121.3, 124.5, 143.7, 144.3, 149.6, 152.4, 153.8, 162.6, 166.0; FAB-MS m/z 300 $(M+H)^+$.

4.1.6.4. *N*6-(2,4,5-Trimethoxybenzoyl)adenine (29). White solid (23%): mp 247.5–249.5 °C; ^{1}H NMR (500 MHz, $CDCl_3$) δ 3.94 (s, 3H), 3.99 (s, 3H), 4.14 (s, 3H), 6.59 (s, 1H), 7.74 (s, 1H), 8.30 (s, 1H), 8.79 (s, 1H), 10.85 (br s, 1H), 11.80 (br s, 1H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 56.5, 56.5, 57.2, 96.5, 110.7, 113.3, 113.8, 143.7, 143.9, 144.2, 152.6, 154.1, 154.7, 162.4, 164.1; FAB-MS m/z 330 $(M+H)^+$; HRMS (ESI) Calcd. For $C_{15}H_{15}N_5O_4Na$: 352.1016, Found: 352.1015.

4.2. BRD4 bromodomain1 TR-FRET assay

BRD4-inhibitory activity was evaluated by means of europium-based LANCE TR-FRET (time-resolved fluorescence resonance energy transfer) assay using a BRD4 bromodomain 1 TR-FRET assay kit (No. 600520, Cayman). We prepared (+)-JQ1 (No. 11187, Cayman), I-BET151 (No. 2220-1, BioVision) and RVX208 (No. 2245, Axon) as positive controls. Plates were read in the time-resolved format by exciting the test compounds at 320 nm and reading emissions at 615 and 665 nm, using a 100 μs delay and a 400 μs read window in Envision (PerkinElmer). Data analysis was performed using the TR-FRET ratio (665 nm emission/615 nm emission).

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