Bioorganic & Medicinal Chemistry Letters 26 (2016) 1365–1370

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry Letters

journal homepage: www.elsevier.com/locate/bmcl

A phenotypic drug discovery study on thienodiazepine derivatives as inhibitors of T cell proliferation induced by CD28 co-stimulation leads to the discovery of a first bromodomain inhibitor

Junichi Endo, Hidemasa Hikawa, Maiko Hamada, Seigo Ishibuchi, Naoto Fujie, Naoki Sugiyama, Minoru Tanaka, Haruhito Kobayashi, Kunio Sugahara, Koichi Oshita, Kazunori Iwata, Shinsuke Ooike, Meguru Murata, Hiroshi Sumichika, Kenji Chiba, Kunitomo Adachi *

Research Unit B, Research Division, Mitsubishi Tanabe Pharma Corporation, 1000, Kamoshida-cho, Aoba-ku, Yokohama, Kanagawa 227-0033, Japan

ARTICLE INFO

Article history:
Received 30 September 2015
Revised 9 December 2015
Accepted 30 January 2016
Available online 2 February 2016

Keywords:
CD28
Phenotypic drug discovery
Chemical biology
BET family
Bromodomain
Immunosuppressant
Anti-cancer

ABSTRACT

A phenotypic screening of thienodiazepines derived from a hit compound found through a binding assay targeting co-stimulatory molecules on T cells and antigen presenting cells successfully led to the discovery of a thienotriazolodiazepine compound (**7f**) possessing potent immunosuppressive activity. A chemical biology approach has succeeded in revealing that **7f** is a first inhibitor of epigenetic bromodomain-containing proteins. **7f** is expected to become an anti-cancer agent as well as an immunosuppressive agent.

BET family proteins (bromodomain-containing protein 2, 3, 4 and T) bind to acetyl-lysine residues in the tail of histones H3 and H4.[1] Bromodomain-containing protein 4 (BRD4) has been reported to relate to regulation of epigenetic expression of genes, cell proliferation, and cell division.[2–5] BET inhibitors have been expected to provide new therapies for cancer and a selective BET inhibitor JQ1 has been reported to show antitumour efficacy in several cancers including the nuclear protein of the testis (NUT) midline carcinoma and multiple myeloma in animals.[6,7] Since the early 2000s we have been searching for novel immunosuppressants showing immunological tolerance focusing on a co-stimulatory molecule on T cells based on a phenotypic drug discovery strategy, obtaining a potent immunosuppressive thienodiazepine compound.[8] A chemical biology approach has successfully elucidated that the compound is a first small molecule inhibiting the protein–protein interaction between histones and BRDs. We herein describe the discovery of novel thienodiazepines and their mode of action (MOA).

At the beginning of 2000s, we were interested in a co-stimulatory molecule, cluster of differentiation 28 (CD28) because the molecule had been elucidated to be involved in immunological tolerance and expected to be a key to development of low-molecular weight immunosuppressants for the treatment of various immunological disorders including rheumatoid arthritis.[9] A high throughput screening focused on binding between B7 proteins on antigen presenting cells and CD28 or CD152 (CTLA-4) on T cells resulted in the discovery of thienodiazepine compound **1** as one of the hit compounds. We immediately executed both in vitro (a cell-based assay using mixed lymphocyte reaction[10]) and in vivo (a mouse collagen-induced arthritis (CIA) model[11]) evaluation of **1**. Interestingly, the compound showed considerably strong therapeutic effects in the CIA model, suggesting that **1** would possess an unidentified mechanism of action in addition to the inhibition of the binding between B7 and CD28 because the inhibitory activity in vitro was weak and we could not explain its in vivo effects. This encouraged us to optimize the compound using a phenotypic assay but not the binding assay. We constructed a cell-based assay system for evaluating the inhibition of T cell proliferation induced by CD28 co-stimulation and started a drug discovery study using this assay system.[12] It is well known that thienodiazepines have

* Corresponding author. Tel.: +81 045 963 7235; fax: +81 045 963 7257.
E-mail address: Adachi.Kunitomo@mc.mt-pharma.co.jp (K. Adachi).

http://dx.doi.org/10.1016/j.bmcl.2016.01.084
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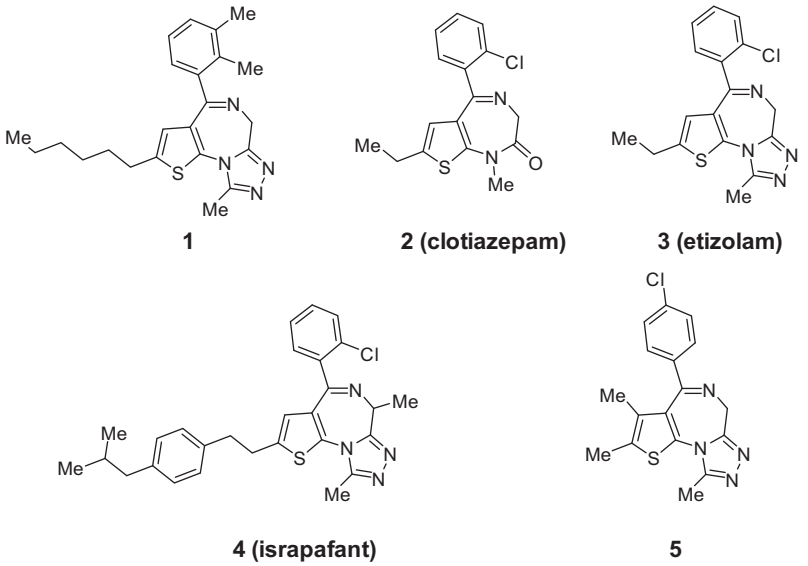
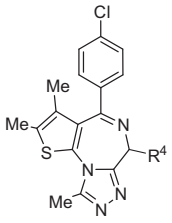


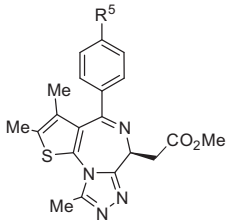
Figure 1. Thienodiazepines.

Table 1
Effect of substitution at the 6-position on inhibition of T cell proliferation induced by CD28 co-stimulation



Compound	R^4	IC_{50} (μM)
5	H	2.5
6a	Me (racemate)	0.39
6b	Et (racemate)	0.34
6c	CH_2Ph (racemate)	0.5
6d	CH_2CH_2Ph (racemate)	2.68
6e	CH_2(2-Py) (racemate)	0.33
6f	CH_2(3-Py) (racemate)	0.4
6g	CH_2(4-Py) (racemate)	0.24
6h	CH_2CO_2Me (*S*)	0.038
6i	CH_2CO_2Me (*R*)	>10
6j	CH_2CO_2Et (*S*)	0.012
6k	CH_2CO_2(*n*-Pr) (*S*)	0.03
6l	CH_2CO_2(*i*-Pr) (*S*)	0.044
6m (JQ1)	CH_2CO_2(*t*-Bu) (*S*)	0.11
6n	CH_2CO_2Bn (*S*)	0.036
6o	CH_2CH_2OMe (*S*)	0.39
6p	CH_2CH_2OH (*S*)	0.58
6q	CH_2CH_2CN (*S*)	0.1

Table 2
SAR of biphenyl derivatives **7a**–**m**

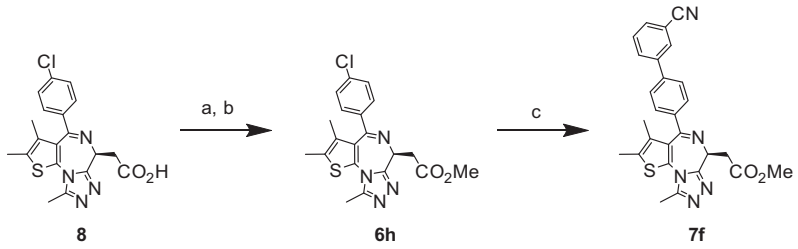


Compound	R^5	IC_{50} (μM)
6h	Cl	0.038
7a	Ph (racemate)	0.22
7b	4-MeOPh	0.053
7c	3-MeOPh	0.036
7d	2-MeOPh	0.19
7e	4-CNPh	0.033
7f	3-CNPh	0.026
7g	2-CNPh	0.18
7h	3-AcNHPh	0.027
7i	$3\text{-}CH_3COPh$	0.015
7j	3-HOPh	0.032
7k	3-MsPh	0.0098
7l	$3\text{-}CF_3OPh$	0.16
7m	3-CN-4-HOPh	0.0025

a variety of biological activity. Anxiolytic drugs, clotiazepam (Rize®, **2**) and etizolam (Depas®, **3**); and an antiasthmatic platelet-activating factor (PAF) antagonist, israpafant (Pafnol™, **4**) have a common thienodiazepine scaffold (Fig. 1). These are our products and we had a large library of this class of compounds. Firstly, we screened **1**-related compounds selected from the library, identifying compound **5** with an IC_{50} of 2.5 μM as the lead.

We focused on the introduction of a substituent at the 6-position of the thienodiazepine ring (Table 1). Introduction of an alkyl or aralkyl group (**6a**–**c**, **6e**–**g**) resulted in a five to ten-fold increase in activity, while phenethyl derivative **6d** retained the activity. We investigated introduction of polar substituents such as ester, ether, alcohol, and cyano groups. Introduction of an (*S*)-methoxycarbonylmethyl group (**6h**) resulted in a drastic increase in activity, while the antipode (**6i**) completely lost the activity, suggesting that (*S*)-configuration at the 6-position would be critical for showing biological activity. Other ester derivatives (**6j**–**n**) with the (*S*)-configuration also showed potent activity, among which ethyl ester **6j** is the most potent. Derivatives with other functionality such as methoxymethyl, hydroxymethyl, and cyanomethyl showed moderate activity (**6o**–**q**). Methyl ester **6h** showed better pharmacokinetic properties than ethyl ester **6j** and we chose **6h** as the lead for further optimization.[13]

We turned our attention to modification of the 4-position of the pendant phenyl group of compound **6h**. We synthesized compounds substituted at the 4-position of the pendant phenyl group of **6h** by another phenyl group with various substituents (Table 2). The introduction of a phenyl group resulted in a five-fold reduction



Scheme 1. Reagents and conditions: (a) PivCl, Et_3N; (b) NaOMe, MeOH (58%); (c) 3-CNPhB$(OH)_2$, Pd$(OAc)_2$, JOHNPHOS, KF, THF (95%).

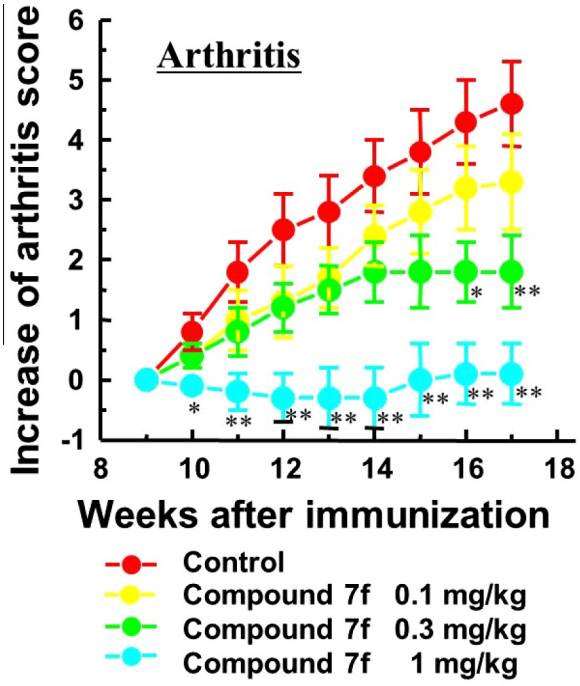


Figure 2. Therapeutic effect of **7f** on chronic-progressing CIA in DBA/1 J mice.[15]

in activity (**7a**); however, methoxyphenyl substituted analogs **7b** and **7c** and cyanophenyl substituted analogs **7e** and **7f** showed comparable activity to **6h**, while 2-substituted phenyl analogs **7d** and **7g** showed considerably reduced activity as compared to **6h**. We next synthesized compounds with a 3-substitued phenyl group at the 4-position of the pendant phenyl group of **6h** (**7h**–**m**). Although a compound with an electron-withdrawing group (CF_3O) showed a four-fold reduction in activity, the other compounds showed potent activity. In particular, compounds **7k** and **7m** were 4- and 25-fold more potent than **6h**, respectively. We evaluated potent inhibitors thus obtained by using the CIA model and toxicity tests, identifying compound **7f**, which showed the most potent therapeutic effects in the CIA model and the least toxicity, as a preclinical candidate to progress into further studies.

Compound **7f** was synthesized as shown in Scheme 1. Chiral acid **8**[14] was esterified to afford **6h**, which was coupled with 3-cyanophenyl boronic acid to give **7f** (Scheme 1).

Compound **7f** was evaluated in vivo and proved to be effective in animal models of autoimmune diseases and transplantation. For example, in a mouse collagen-induced arthritis model, **7f** demonstrated a potent therapeutic effect at an oral dose of 0.3 mg/kg or higher (Fig. 2).

We started a research program to elucidate the MOA of **7f** using Jurkat cells. We synthesized several chemical probes including **9** and **10** and succeeded in identifying the target protein of **7f** by using a biotin conjugate **10** (Fig. 3).

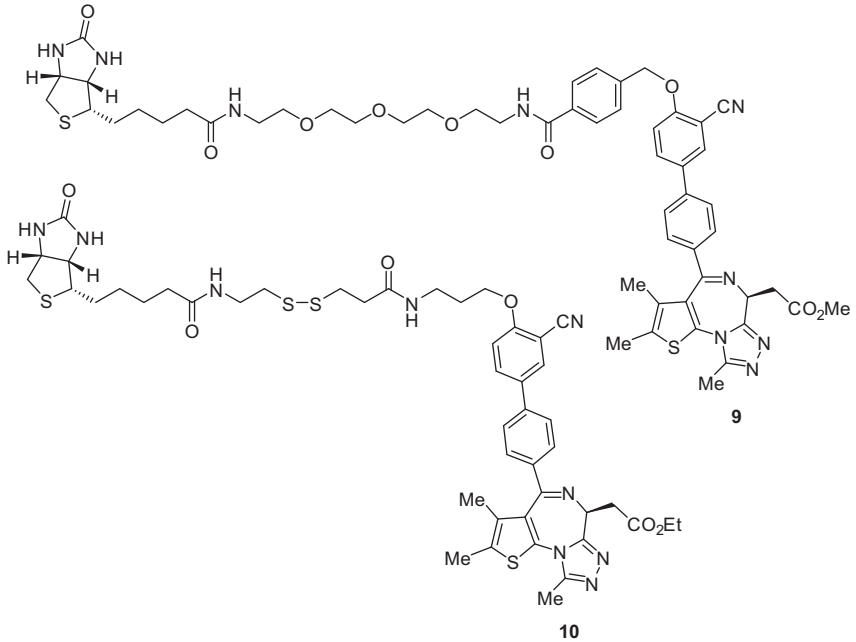
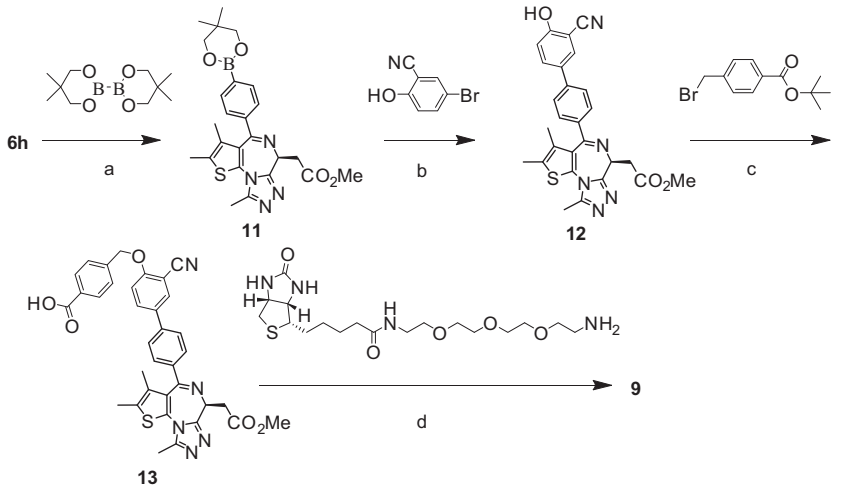
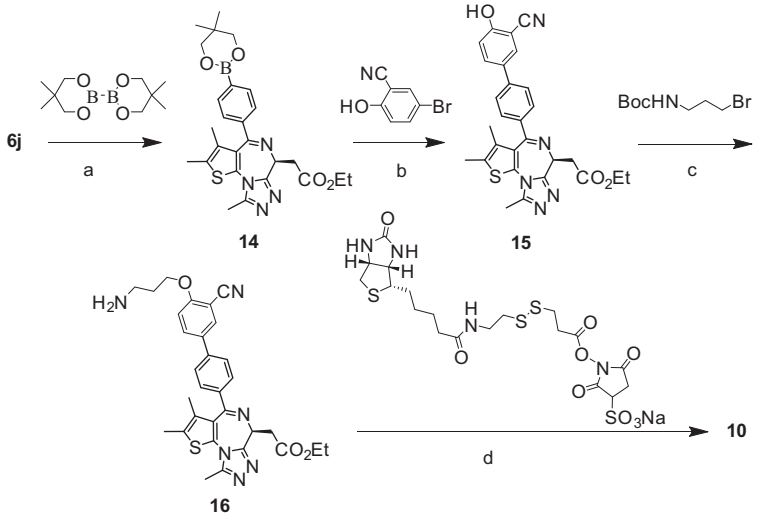


Figure 3. Chemical probes for the pull down assay.



Scheme 2. Reagents and conditions: (a) $PdCl_2(PCy_3)_2$, KOAc, dioxane (100%); (b) $PdCl_2(PPh_3)_2$, Na_2CO_3, THF, H_2O (75%); (c) K_2CO_3, DMF, then TFA, CH_2Cl_2 (92%), (d) EDC HCl, HOBt, Et_3N, DMF (29%).



Scheme 3. Reagents and conditions: (a) $PdCl_2(PCy_3)_2$, KOAc, dioxane (100%); (b) $PdCl_2(PPh_3)_2$, Na_2CO_3, THF, H_2O (52%); (c) Cs_2CO_3, MEK, then TFA, CH_2Cl_2 (28%), (d) THF, DMSO (17%).

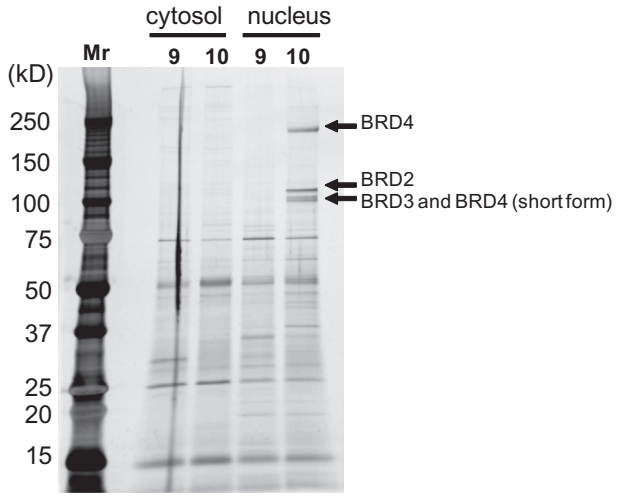


Figure 4. Results of the pull down assay.[16]

Chemical probes **9** and **10** were synthesized as outlined in Schemes 2 and 3, respectively.

For elution of the specific binding molecules to the compound, we used reducing reagent tris(2-carboxyethyl)phosphine (TCEP). Compound **10** has a disulfide bond in the linker, while compound **9** does not. As a result, the target molecules were successfully eluted from the nucleus fraction using compound **10** and avidin-resin. We identified nuclear proteins, bromodomain-containing proteins BRD2, BRD3, and BRD4 as the target molecules (Fig. 4).[16]

Next, we examined whether addition of **7f** competitively inhibits the pull down of the BRDs induced by **10**/avidin-resin. As a result, **7f** inhibited the pull down dose-dependently but the antipode of **7f** (anti-**7f**) did not inhibit the pull down, confirming that **7f** binds to the BRDs and is a first inhibitor against BRDs (Figs. 5).[16] It has been also verified that **7f** inhibits the binding between BRD4 and an acetylated histone peptide (AcH4) with an IC_{50} value of 33.9 nmol/L (Fig. 6) (see Supplementary material). Based on these findings, **7f** is speculated to show its therapeutic effect on the chronic-progressing CIA by inhibiting T cell activation and proinflammatory cytokine production which are mediated by the BRD-acetylated histone interaction.

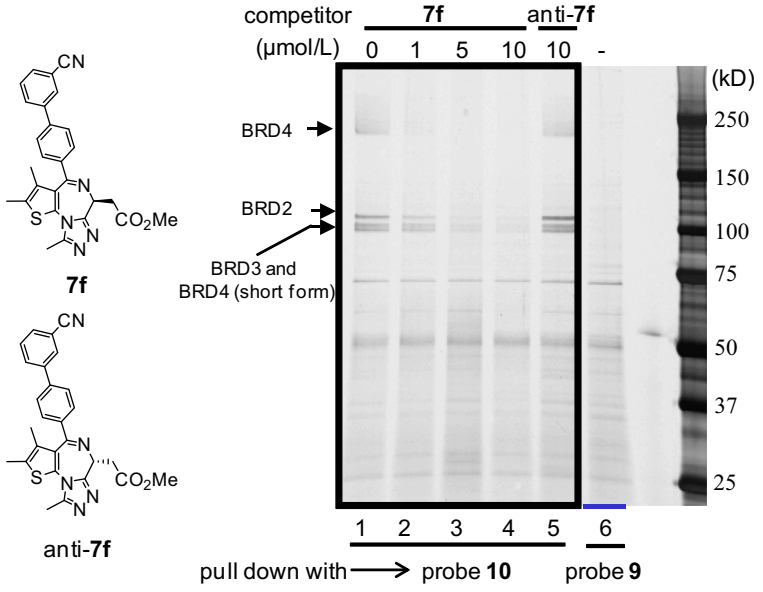


Figure 5. Inhibition of the pull down by **7f** and anti-**7f**.

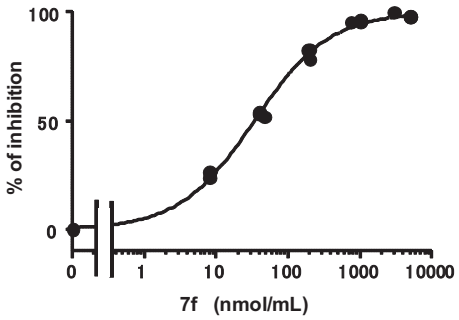


Figure 6. Inhibition of BRD4-AcH4 binding by **7f**.

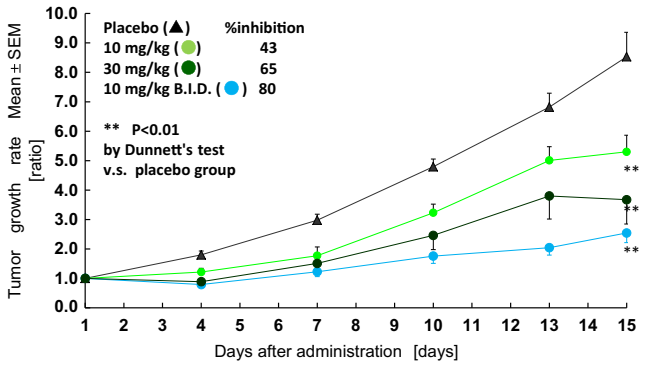


Figure 7. Anti-tumor activity of **7f**.[18]

Table 3
Effects of compound **7f** on cell survival[17]

Cell line	Tissue type	GI_{50} (μM)
M-1	Mouse myeloid leukemia	0.003
HL-60	Premyelocytic leukemia	0.007
MV4-11	CML (Chronic Myeloid Leukemia)	0.019
Daudi	Burkitt's lymphoma	0.067
U-937	Burkitt's lymphoma	0.829
RPMI-8226	Myeloma B-cell type lymphoma	0.069
MOLT-4	ALL (Acute Lymphoblastic Leukemia)	0.106
B16	Mouse melanoma	0.080
Kim-1	Hepatocellular cancer	0.104
A-2780	Ovarian cancer	0.130
SKOV-3	Ovarian cancer	5.27
PC-3	Prostate cancer	0.199
LNCap	Prostate cancer	0.201
EBC-1	NSCLC (Non Small Cellular Lung Cancer)	0.207
A549	NSCLC	>10
LN-18	Brain cancer	0.207
U-87MG	Brain cancer	>10
Hela	Cervical cancer	0.274
Colo-205	Colorectal cancer	0.484
LLC	Lewis lung carcinoma	1.50

Since it turned out that compound **7f** specifically binds to BRD2/3/4 proteins and inhibits their binding to acetylated histones, we started investigating inhibitory activity of 7**f** against the in vitro proliferation of tumor cell lines.[17] 7**f** exhibited inhibitory activity on the in vitro growth of wide variety of tumor cell lines with submicromolar potency, especially strong activity against leukemia, lymphoma and multiple myeloma cell lines. In solid tumors, prostate cancer, lung cancer and colorectal cancer cell lines showed high sensitivity against 7**f** (Table 3).

Compound **7f** was evaluated for its in vivo anti-tumor efficacy by using xenograft of human promyelocytic leukemia HL-60 cell line implanted onto nude mice (Fig. 7).[18] Significant inhibition of relative tumor growth was achieved to 43%, 65% and 80% in the mice receiving 10, 30 mg/kg and 10 mg/kg twice a day of **7f**, respectively (p-value <0.01), suggesting that **7f** would be useful as an anticancer agent.

In summary, a phenotypic assay targeting the inhibition of T cell proliferation induced by CD28 co-stimulation led to the discovery of a novel immunosuppressive thienodiazepine compound **7f**, which has proved to be a first BRD inhibitor by using a chemical biology method and is expected to become not only an immunosuppressive agent but also an anticancer agent.

Acknowledgments

We thank Mr. Hikaru Nonaka and Ms. Junko Wada for constructing a high through-put screening system (binding between B7 and CD28/CTLA-4) and executing the assay.

Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmcl.2016.01.084.

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11. The effects of compounds on mouse collagen-induced arthritis were evaluated as follows. DBA/1J mice were immunized with type II collagen twice between 21 days interval. Compounds were orally administered to DBA/1J mice for 3 weeks from the day of secondary immunization with type II collagen. Arthritis score was judged at 21–42 days after primary immunization according to the following criteria. Score 0, no change; score 1, edema at one joint; score 2 edema at two or more joints, or mild edema throughout the limbs; score 3, severe edema throughout the limbs; score 4, severe edema throughout the limbs and ankylosis.
12. Inhibition of T cell proliferation induced by CD28 co-stimulation was evaluated as follows. Splenic T cells from BALB/c mice were stimulated with anti-CD28 Ab (10 ng/mL) and phorbol 12-myristate 13-acetate (PMA, 250 ng/mL) in the presence of compounds. T cell proliferation after 24 h culture was determined by [methyl-3*H*]thymidine incorporation assay. IC_{50} values were calculated by linear regression.
13. The pharmacokinetic parameters for **6h** and **6j** in rats are as follows. **6h** (3 mg/kg, iv), $AUC_{0-\infty}$: 1185 ng·h/mL; CL: 2568 mL/h/kg; V_{ss}: 4584 mL/kg, **6h** (10 mg/kg, po), C_{max}: 663 ng/mL; T_{max}: 0.83 h; $AUC_{0-\infty}$: 2674 ng h/mL; BA: 67.7%. **6j** (3 mg/kg, iv), $AUC_{0-\infty}$: 1081 ng·h/mL; CL: 2799 mL/h/kg; V_{ss}: 1324 mL/kg, **6j** (10 mg/kg, po), C_{max}: 1293 ng/mL; T_{max}: 0.5 h; $AUC_{0-\infty}$: 1778 ng·h/mL; BA: 49.3%.
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15. The therapeutic effect of **7f** on chronic-progressing CIA was evaluated as follows. DBA/1J mice were immunized with bovine type II collagen and Freund's complete adjuvant once. After 9 weeks of the immunization, **7f** was orally administered to the mice for 8 weeks. Arthritis score were determined weekly during administration periods. Each symbol represents the mean ± SEM of arthritic score of 12 animals. Statistical significance versus arthritis control group was calculated by Steel method. (*: p <0.05, **: p <0.01.)
16. The pull down assay method (Fig. 4) and the competition experiment (Fig. 5) are as follows. The compound **9** or **10** was mixed with NeutrAvidin-resin (Thermo Scientific; Pierce Protein Biology Products, Rockford, IL) at rt for 30 min with vortex. These complexes were washed with PBS and then incubated with PMA/ionomycin activated Jurkat cell lysate (cytosol and nucleus fractions), which were extracted by the Nuclear extract kit (Sigma–Aldrich, St Louis, MO), at 4 °C, for 2 h with rotation. After washing the complexes with TNE buffer (10 mM Tris–HCl pH 7.5/1% NP-40/0.15 M NaCl/1 mM EDTA), the complexes were incubated with 20 μmol/L of reducing reagent TCEP (Thermo Scientific; Pierce Protein Biology Products, Rockford, IL) at rt for 15 min with vortex. Removing the NeutrAvisin-resin by centrifugation, the supernatants were analyzed by SDS–PAGE. In the competition experiment, compound **7f** or anti-**7f** was added in the Jurkat cell lysate before the pull down assay.
17. Tumor cells were exposed 72 h at 37 °C, 5% CO_2 to **7f** at various concentrations. At the end of incubation, cell viability and proliferation were quantified by using a tetrazolium salt (WST-8) based colorimetric assay. We created dose-growth inhibition curve, and calculate GI_{50} values (geometric mean value) and 95% confidential interval by non-linear regression analysis.
18. **7f** was orally administered for 14 days at a dose of 10, 30 mg/kg/day once a day or 10 mg/kg twice a day. The tumor mass was measured to calculate the relative tumor growth rate.