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Discovery, Design, and Optimization of Isoxazole Azepine BET Inhibitors

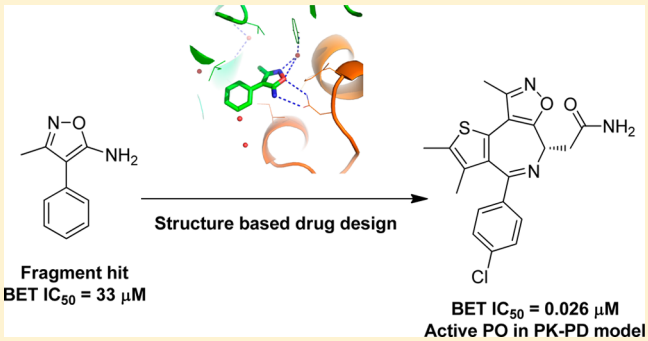
Victor S. Gehling,*[,†] Michael C. Hewitt,*[,†] Rishi G. Vaswani,[†] Yves Leblanc,[†] Alexandre Côté,[†] Christopher G. Nasveschuk,[†] Alexander M. Taylor,[†] Jean-Christophe Harmange,[†] James E. Audia,[†] Eneida Pardo,[†] Shivangi Joshi,[†] Peter Sandy,[†] Jennifer A. Mertz,[†] Robert J. Sims, III,[†] Louise Bergeron,[†] Barbara M. Bryant,[†] Steve Bellon,[†] Florence Poy,[†] Hariharan Jayaram,[†] Ravichandran Sankaranarayanan,[‡] Sreegouri Yellapantula,[‡] Nandana Bangalore Srinivasamurthy,[‡] Swarnakumari Birudukota,[‡] and Brian K. Albrecht[†]

[†]Constellation Pharmaceuticals, 215 First Street, Suite 200, Cambridge, Massachusetts 02142, United States

[‡]Jubilant Biosys Limited, #96, Industrial Suburb, Second Stage, Yeshwantpur, Bangalore 560 022, India

Supporting Information

ABSTRACT: The identification of a novel series of small molecule BET inhibitors is described. Using crystallographic binding modes of an amino-isoxazole fragment and known BET inhibitors, a structure-based drug design effort lead to a novel isoxazole azepine scaffold. This scaffold showed good potency in biochemical and cellular assays and oral activity in an in vivo model of BET inhibition.



KEYWORDS: *BET inhibitors, bromodomain, isoxazoles, fragments, MYC*

The bromodomain and extra-C terminal domain (BET) family of proteins is made up of chromatin adaptors that contain tandem bromodomains that recognize specific acetylated lysine residues in the N-terminal tails of histones. Members of the BET family, including BRD2, BRD3, BRD4, and BRDT, regulate gene expression by recruiting transcriptional regulators to specific genomic locations.[1] The proteins in the BET family are known to control expression of genes critical for proper cell growth, and reduction of their levels or inhibition of their activity has profound effects on cell proliferation and viability. Small molecule BET inhibitors initially appeared in the patent literature[2] and were followed by publications detailing activity in models of cancer and inflammation.[3,4] The initial reports indicated that BET inhibition led to antiproliferative effects in a BRD4-NUT mediated midline carcinoma.[3] In addition, it has been reported that BET inhibition suppresses a subset of NF-κB dependent inflammatory genes following LPS stimulation in macrophages, with concomitant efficacy in a mouse model of endotoxic shock.[4] The mechanistic link between BET activity and cancer was further developed when several groups showed that small molecule inhibition of BET reduced transcription of the MYC oncogene,[5] resulting in cell cycle arrest and activity in vivo in models of leukemia and lymphoma.[6,7] The demonstrated efficacy of BET inhibitors in cancer xenograft and immunology models, combined with their ability to modulate a compelling subset of genes prompted us to initiate a program to discover small molecule inhibitors of BET.[8−20]

Our drug discovery efforts began with a fragment screen that uncovered several compounds with potencies in the micromolar (μM) range. Several of these fragments were cocrystallized with BRD4 BD1 to give high-resolution structures. Of the cocrystal structures obtained, the amino-isoxazole **1**/BRD4-BD1 complex was of particular interest. It was observed that the amino-isoxazole **1** mimicked the key interactions found between BRD4-BD1 and the endogenous acetylated lysine binding partner (Figure 1).

Comparison of the binding modes of **1** and the reported diazepine inhibitor JQ1[3] (**2**) showed that both **1** and **2** made similar hydrogen bonding contacts with the asparagine residue critical for recognition of the acetyl lysine substrate and suggested that an isoxazole could serve as a replacement for the triazole of **2**. Subsequent to our initial fragment work with amino isoxazole **1**, the 3,5-dimethyl isoxazole motif was disclosed as a preferred binding motif for bromodomains (see refs 9−11 and 16). Compounds **1** and **2** also filled a part of the

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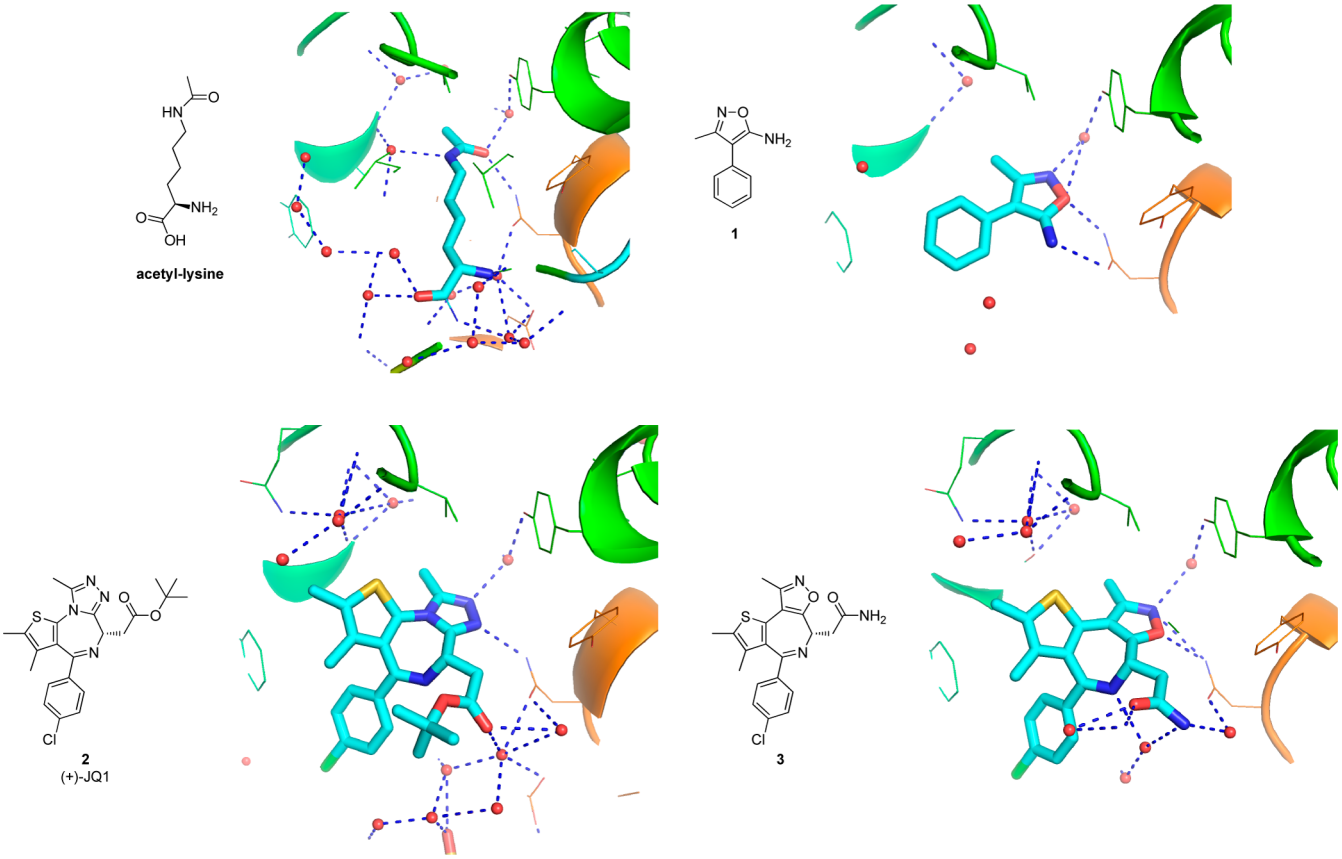
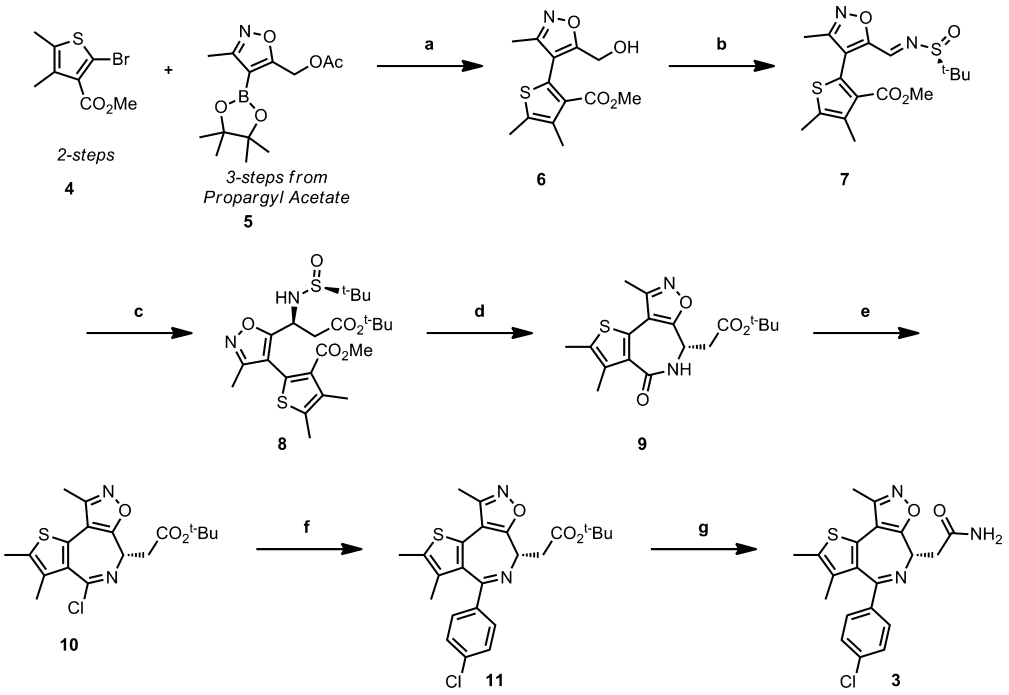


Figure 1. Crystal structures of acetyl-lysine,[21] amino isoxazole **1**,[22] JQ1 (**2**),[3,23] and isoxazole azepine **3**[24] in BRD4 BD1.

Scheme 1. Synthesis of Isoxazole Azepine 3[a]



[a]Reagents: (a) (i) $Pd_2(dba)_3$, SPhos, K_3PO_4, *n*-BuOH, 100 °C; (ii) MeONa (cat.), MeOH (88% over 2 steps); (b) (i) Swern oxidation, (ii) $Ti(OEt)_4$, (*S*)-*t*-butyl sulfinylamide (91% over 2 steps); (c) 2-(*tert*-butoxy)-2-oxoethyl)zinc(II) chloride, NMP, −10 °C (89%, d.r. 7:1 to 5:1); (d) (i) HCl, MeOH, (ii) *i*-PrMgBr, THF (80% over 2 steps); (e) PCl_5, CH_2Cl_2 (65%); (f) $Pd(PPh_3)_4$, K_2CO_3, 4-chlorophenylboronic acid, toluene/water (10:1), 95 °C (82%); (g) (i) TFA (50 equiv), $CHCl_3$, 36 °C, (ii) HATU, NH_4Cl, Et_3N, CH_2Cl_2 (80% over 2 steps).

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acetyl binding pocket with a methyl group from either the isoxazole or triazole moieties and acted as acetyl lysine mimics. In addition, the LEAN of **1** was calculated to be 0.34 and binding efficiency 25.7, which made the fragment an excellent starting point for our drug discovery efforts.[25−28] We rationalized that incorporating the isoxazole motif into an azepine scaffold would provide a concave shape similar to **2** and afford compounds with increased potency against BRD4 as compared to isoxazole **1**. Herein, we report the genesis, synthesis, structure−activity relationships (SARs), biochemical potency, cellular potency, and in vivo activity of a series of isoxazole azepine BET inhibitors.

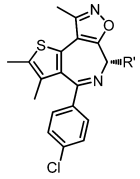
Synthesis of compound **3** (Scheme 1) began with bromothiophene **4** and isoxazole boronic ester **5**, both readily synthesized in three steps from commercially available starting materials (see Supporting Information for experimental details). Union of **4** and **5** via Suzuki reaction and subsequent acetate deprotection afforded primary alcohol **6**. Oxidation and imine formation with (*S*)-*tert*-butyl sulfinamide[29] formed sulfinyl imine **7**. The addition of the Reformatsky enolate of *tert*-butyl acetate to imine **7** afforded sulfinamine **8** and established the requisite stereocenter, albeit with modest diastereoselectivity (d.r. 7:1 to 5:1). The sulfinyl amine was then deprotected under acidic conditions, and subsequent treatment with *i*-PrMgBr provided lactam **9**. Chlorination of **9** with PCl_5 afforded imidoyl chloride **10**, which was converted to azepine **11** under standard Suzuki reaction conditions. Deprotection of the *tert*-butyl ester and amide bond formation provided desired compound **3**.

The first synthetic entry in our SAR table was the direct isoxazole analogue of JQ1 (**2**), *tert*-butyl ester intermediate **11**, which showed modest potency in our biochemical assay and poor translation into cells (Table 1). The unexpected loss of potency of **11** prompted us to focus on replacing the suboptimal *tert*-butyl protecting group with a variety of amides and amide isosteres. The first amide analogue we made was the simple primary carboxamide **3**, which showed excellent biochemical and cellular potency.

The next entries in Table 1 were ethyl amide **12**, which showed good cellular and biochemical potency, and enantiomer **13**. Compound **13** was over 50-fold less potent than **12**, showing that activity was strongly dependent on the stereochemistry of the side chain attached to the azepine core.[30]

Further exploration of the side-chain substituents was performed to determine whether additional potency could be gained in this area. Our attempts to change the nature or location of the hydrogen bond donor were successful biochemically, as the carbamate (**14**) and reversed amide (**15**) retained biochemical potency. Other modifications of the amide, including replacing the amide with a heterocycle (**16** and **17**) or incorporation of an α-methyl group (**18**) led to less cellular potency, although the changes were tolerated biochemically. With the preliminary SAR pointing to the superiority of the carboxamide side-chain, we obtained a crystal structure of compound **3** (Figure 1). Like the previously reported dimethylisoxazoles (PDB ID's 4GPJ,[9] 3SVF,[10] and 3SVG[11]), compound **3** also formed a hydrogen bond with Asn140 and a water mediated hydrogen bond with Tyr 97. At 3.2 Å, the H-bonding distance between the isoxazole and Asn140 was similar to that observed in the literature structures (range 2.9−3.2 Å). The ring system below the isoxazole in compound **3** was within van der Waal distance of the Ile146 residue and wrapped around this residue, which is also a common feature of the reported bromodomain inhibitors.

Table 1. Isoxazole Azepine Side-Chain SAR[31,32]



Compound	R'	BRD4 BD1 IC_{50} (μM)[a]	Raji IC_{50} (μM)[b]
1	-	33[c]	n.t.
11		0.29 ± 0.07	2.1 ± 0.05
3		0.026 ± 0.01	0.14 ± 0.05
12		0.036 ± 0.01	0.32 ± 0.02
13 (*R* stereochem.)		2.1[c]	n.t.
14		0.086 ± 0.01	1.9 ± 0.2
15		0.19 ± 0.06	2.2 ± 0.05
16		0.20 ± 0.05	3.6 ± 0.5
17		0.19 ± 0.04	2.1 ± 0.2
18[d]		0.25 ± 0.01	0.71 ± 0.1
JQ1[3]	-	0.024 ± 0.01	0.069 ± 0.03
I-BET151[12-14]	-	0.018 ± 0.01	0.13 ± 0.02
I-BET762[4]	-	0.12 ± 0.1	0.19 ± 0.01

[a]An average of ≥3 determinations, with standard deviation (SD) reported. [b]An average of ≥2 determinations, with SD reported. Our MOA cell assay used suppression of MYC expression in Raji cells; see Supporting Information for details. Raji cells were chosen because they are a MYC-dependent cancer cell line, showing potent growth inhibition and MYC downregulation upon BET inhibitor treatment. [c]Single measurement, no SD reported. [d]Compound **18** had 4-cyanophenyl instead of 4-Cl-phenyl substitution. n.t. = not tested.

Modifications to the 4-chlorophenyl ring was the next SAR vector we examined, with the goal of modulating the biophysical and 3-dimensional properties of our analogues, while maintaining the potency we had achieved in analogues such as **3** (Table 2). We began with a chlorine scan around the aromatic ring. As might be predicted from the crystal structure, *para*- or *meta*-chlorine substitution (**3** and **19**) afforded similarly potent compounds in both biochemical and cellular assays. *ortho*-Chloro substitution (**20**) led to a 10-fold drop in biochemical potency, perhaps because this substitution induced an unfavorable twist between the two unsaturated systems. However, the *ortho*-methyl and *para*-chloro substitutions (**23**) were tolerated, leading to a compound with potency similar to that of the parent, suggesting that the *ortho*-chloro substituent was not tolerated due to an unfavorable electrostatic interaction, rather than a steric one. Other aromatic systems were tolerated (**21** and **22**) but did not appear to offer any clear

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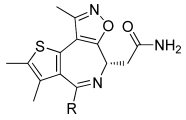
Table 2. Aromatic SAR



Compound	R	BRD4 BD1 IC_{50} (μM)[a]	Raji IC_{50} (μM)[b]
3		0.026 ± 0.01	0.14 ± 0.05
19		0.074 ± 0.03	0.47 ± 0.2
20		0.25 ± 0.05	1.9 ± 0.6
21		0.029 ± 0.01	0.18 ± 0.07
22		0.033 ± 0.02	0.091 ± 0.09
23		0.042 ± 0.01	0.16 ± 0.05
24		0.072 ± 0.02	0.24 ± 0.03
25		0.073 ± 0.01	0.38 ± 0.09
26		2.5 ± 0.9	n.t.
27		5.7 ± 2	n.t.
28		0.30 ± 0.05	1.5 ± 0.1
29		1.1 ± 0.5	n.t.
30		6.2 ± 1	n.t.

[a]An average of ≥3 determinations, with SD reported. [b]An average of ≥2 determinations, with SD reported. Our MOA cell assay used suppression of MYC expression in Raji cells; see Supporting Information for details. Raji cells were chosen because they are a MYC-dependent cancer cell line, showing potent growth inhibition and MYC downregulation upon BET inhibitor treatment. n.t. = not tested.

advantages. Replacing the aromatic ring with 2-pyridine (**24**) or amino-pyridine (**25**) was tolerated, but substitution with a pyrimidine (**26**) was not. Replacement of the aromatic ring with a saturated system and polar saturated system, as in **28**, **29**, or **30**, was also not tolerated in the highly lipophilic WPF shelf region of the binding site.

We next profiled a selection of our isoxazole azepines in several in vitro ADME screening assays (Table 3). Universally the compounds showed good stability in human liver microsomes (HLM) and were generally stable in rat liver microsomes (RLM). The only exception was compound **21**, which showed very high clearance in RLM, likely due to the completely exposed phenyl ring. In addition, these compounds displayed high plasma protein binding in human plasma. The single-point CYP inhibition also showed negligible inhibition across the series. Overall, the profiles of compounds **3** and **22** supported further profiling in rat PK experiments.

Of the two compounds profiled in vivo in rats, compound **3** showed the best profile: a $t_{1/2}$ of 1.4 h, V_{ss} of 2.45 L/kg, and a bioavailability of 31% (Table 3). For compound **22**, we had seen instability in rat plasma in our PPB assay, which probably contributed to the higher in vivo clearance for this compound and might explain the discontinuity with the lower in vitro

Table 3. ADME and PK for Selected Compounds

compd	**3**	**22**	**21**	**25**	**3**	**3**
mCL_{int}[a]	140					
rCL_{int}[b]	27	8.9	160	18		
dCL_{int}[c]	11					
hCL_{int}[d]	5.5	4.6	2.4	5.4		
PPB (%)						
mouse	98.1					
rat[e]	99.2	n.a.	n.a.	n.a.		
human	99.4	95.9	98.2	99.4		
CYP 3A4 (%)[f]	20	0	1	20		
PK species	rat				dog	mouse
CL_{int} (L/h/kg)[g]	2.77	4.35			0.32	
V_{ss} (L/kg)[g]	2.45	2.22			1.27	
$t_{1/2}$ (h)[g]	1.39	0.393			3	
AUC (ng h/mL)[h]	605	504				723
F (%)[h]	31	44				

[a]Mouse liver microsomes clearance (μL/min/mg). [b]Rat liver microsomes clearance (μL/min/mg). [c]Dog liver microsomes clearance (μL/min/mg). [d]Human liver microsomes clearance (μL/min/mg). [e]n.a. = not applicable, compounds not stable in rat plasma. [f]In vitro inhibition of cytochrome P450 3A4 isoforms, % inhibition at 10 μM. [g]IV 1 mg/kg. [h]PO 5 mg/kg.

microsomal clearance (compared to **3**). Compound **3** was also profiled in vivo in mouse, as our PD assay was to be run in mouse and dog. We saw adequate exposure in mouse, which gave us confidence we could use our PD model to test target engagement. As we were also interested in translation of this chemotype into higher species, we were pleased that compound **3** showed excellent dog PK, with low clearance (0.32 L/h/kg), moderate volume (1.3 L/kg), and a half-life of 3 h.

Our target engagement assay was based on the documented binding of BRD4 at the MYC locus and known downregulation of MYC transcription following small molecule BET inhibition.[6,7] On the basis of the PK of **3** in mouse and its high clearance in MLM, doses of 10, 30, and 100 mg/kg PO were chosen for the MYC PK-PD experiments. As shown in Figure 2a, a dose-dependent decrease in MYC expression was observed, with highest suppression at 100 mg/kg. Additionally, MYC suppression correlated with the amount of compound in the plasma and tumor, with a ∼75% reduction in MYC levels with nearly 5 μM compound in the tumor at 4 h after a 100 mg/kg dose. To better understand the dynamics of MYC expression upon BET inhibition, a time course experiment was run, which showed that dosing with **3** at 100 mg/kg led to inhibition of MYC expression out to 8 h (Figure 2b). After 8 h, MYC levels rebounded to vehicle levels. This was consistent with elimination of **3** from both the tumor and plasma and suggested that MYC expression could recover rapidly upon compound elimination.

In conclusion, we have described the identification of fragment **1** as a micromolar inhibitor of the BET family of proteins and the elaboration of **1** into novel isoxazole azepine **3**. Compound **3** is a potent and selective[33] BET inhibitor in biochemical and cellular assays, has a rat and dog pharmacokinetic profile suitable for in vivo experiments, and inhibits MYC mRNA expression in vivo after PO dosing in a dose-dependent manner. Further studies detailing the optimization and development of **3** and related compounds will be reported in due course.

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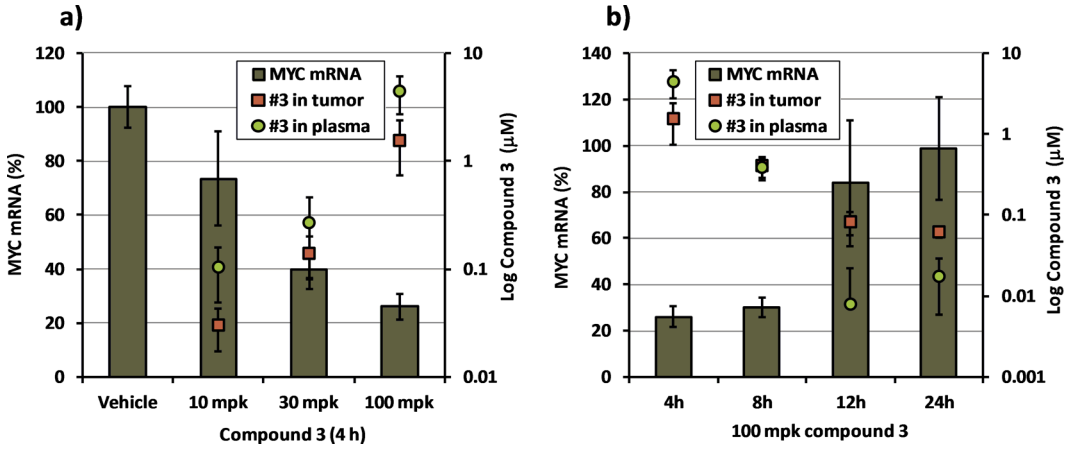

Figure 2. MYC mRNA expression in Raji tumor in mice with compound **3** dosed 10 to 100 mpk PO (a) and a time course with compound **3** at 100 mpk PO (b).

■ ASSOCIATED CONTENT

Supporting Information

Synthetic procedures, assay details, PK–PD assay description, and DSF selectivity profile of compound **3**. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

*(V.S.G.) Tel: 617-714-0540. E-mail: victor.gehling@constellationpharma.com. (M.C.H.) Tel: 617-714-0569. E-mail: mike.hewitt@constellationpharma.com.

Notes

The authors declare no competing financial interest.

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■ DEDICATION

Dedicated to Professor Robert M. Williams on the occasion of his 60th birthday.

■ ABBREVIATIONS

ADME, absorption, distribution, metabolism, excretion; COMU, (1-cyano-2-ethoxy-2-oxoethylidenaminooxy)-dimethylamino-morpholino-carbenium hexafluorophosphate; CYP, cytochrome P450; dba, 1,5-diphenyl-1,4-pentadien-3-one; HATU, *O*-(7-azabenzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate; HLM, human liver microsomes; LPS, lipopolysaccharide; mg/kg, milligram per kilogram; MLM, rat liver microsomes; NMP, 1-methyl-2-pyrrolidinone; NF-κB, nuclear factor kappa-light-chain-enhancer of activated B cells; Pin, 2,3-dimethylbutane-2,3-diol; PK–PD, pharmacokinetic–pharmacodynamic; RLM, rat liver microsomes; SAR, structure–activity relationship; SPhos, 2-dicyclohexylphosphino-2′,6′-dimethoxybiphenyl; TFA, trifluoroacetic acid

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(32) The in vitro potency data for the three literature reference compounds (JQ1, I-BET151, and I-BET762) was generated in our biochemical and cellular assays, under identical conditions to the test compounds.
(33) See Supporting Information for selectivity data of compound **3**.