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Discovery of EBI-2511: a highly potent and orally active EZH2 inhibitor for the treatment of non-Hodgkin lymphoma

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KEYWORDS Benzofuran, EZH2, Lymphoma, Scaffold hopping

ABSTRACT: A novel series of benzofuran derived EZH2 inhibitors were discovered through scaffold hopping approach based on the clinical compound of EPZ-6438. Further rational SAR exploration and optimization led to the discovery of more potent EZH2 inhibitors with oral bioavailability in mice and rats. A lead compound **EBI-2511**(compound **34**) demonstrated excellent *in vivo* efficacy in Pfeiffer tumor Xenograft models in mouse and is under preclinical development for the treatment of cancers associated with EZH2 mutations.

Enhancer of zeste homolog 2 (EZH2) is a subunit of the polycomb-repressive complex 2 (PRC2), which belongs to a class of methyltransferases involved in divergent biological processes, especially in chromatin remodeling and epigenetic silencing[1-3]. Although PRC2 contains other subunits such as RbAp48, EED, Suz12 *etc*, EZH2 is a major catalytic component in transferring three methyl groups to lysine 27 of histone 3 (H3K27), which subsequently leads to gene specific silencing, which includes some tumor suppressor genes[4]. Overexpression or activating mutations of EZH2 (e.g, A677G, Y641F, Y641N,) have been implicated in a variety of cancers including non-Hodgkin's lymphoma and some solid tumors[5-6]. As a result, EZH2 has been pursued by a number of companies as a potential target for small-molecule anti-cancer therapeutics[7].

There are several reported EZH2 inhibitors with different scaffolds in the public domain (**Figure 1**). The first EZH2 inhibitor which advanced into clinical trials, Tazemetostat (EPZ-6438)[8-9] with a biaryl structure, was developed by Epizyme and is currently undergoing Phase II clinical trials for the treatment of a variety of malignant cancers such as diffuse large B-cell lymphoma (DLBCL), follicular lymphoma (FL), and INI1-negative tumors. Subsequently, GSK-2816126[10] (Phase II) and CPI-1205[11] from Constellation (Phase I) bearing a similar indole core were pushed into clinical trials as well. It was noted that DS-3201(structure undisclosed), developed by Daiichi Sankyo, recently entered phase I clinical trials[12]. In a preclinical study, Pfizer reported its EZH2 inhibitor with the new chemotype of dihydroisoquinolin-1(2H)-one[13]. Interestingly, almost all EZH2 inhibitors have been reported to have similar and unique pyridone moieties which are necessary to improve binding to the EZH2 domain in a SAM-competitive manner. However, pyridone oxidation is a potential metabolic route for pyridone-containing EZH2 inhibitors[14-15]. Therefore, all three clinical molecules, as described above, had poor pharmacokinetic (PK) profiles. For GSK-2816126, its recommended administration for clinical trials was by intravenous (iv) infusion. Replacement of pyridone with other moieties has been an active area of investigation done in an effort to avoid metabolic issues[15]. Unfortunately, all of these efforts resulted in substantial loss of enzymatic or cellular activity against EZH2. As a result, in order to create a sufficient exposure in animal models or patients, a much higher dosage for EZH2 inhibitors was required. For example, the clinical dose of EPZ-6438 was increased to 800mg po BID[16]. Therefore, developing more potent EZH2 inhibitors with improved PK/PD profile was warranted. Herein, we report a highly potent and orally efficacious EZH2 inhibitor **EBI-2511,** which contains a novel benzofuran scaffold.

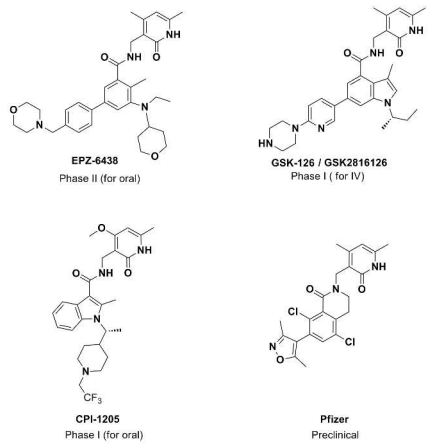


Figure 1. Structures of representative EZH2 inhibitors

On the basis of the reported EPZ-6438's SAR from the literature[8] we were able to deduce that the pyridone fragment with amide bond played a key role in making critical interactions with the EZH2 domain. While previous work had indicated that, this moiety would be difficult to replace. Surprisingly, excluding a left side chain of substituted phenyl, bromide analogue **1** is only 5-6 fold less potent than its parent compound in *in vitro* enzymatic activity against A677G (see **Table 1**). However, it was a less complex structure with lower molecular weight which made it a superior starting point for further modification. We envisioned that some analogues bearing 6/5 fused ring heteroarenes such as benzofuran or benzothiophene would maintain similar binding interactions and show some activity against A677G (**Figure 2**). To the best of our knowledge, these novel scaffolds have not been explored for the development of EZH2 inhibitors[17].

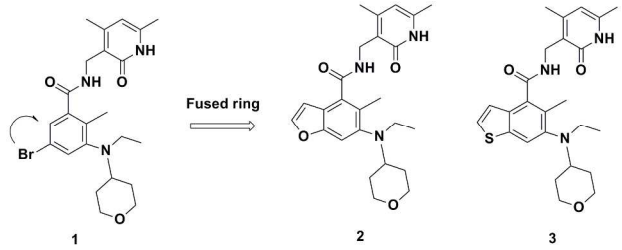
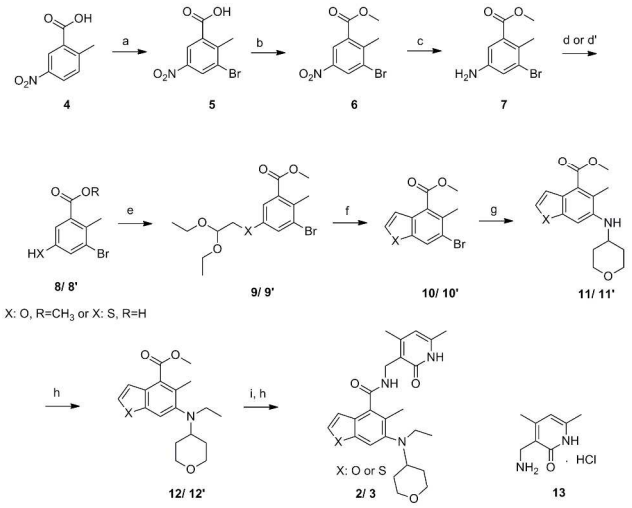


Figure 2. Initial design

To test this theory, compound **2** and **3** were synthesized as shown in Scheme 1. Consistent with previous work[18], 2-methyl-5-nitrobenzoic acid **4** undergoes a selective bromination at the meta-position of the phenyl ring to give compound **5**. After subsequent esterification and reduction, the key intermediate aniline **7** was obtained. The amino group of this intermediate was transferred to a hydroxyl or thiol via diazotization. Phenol **8** or phenthiol **8'** were reacted with 2-bromo-1,1-diethoxyethane to give the alkylated products **9** or **9'** , which was rapidly followed by a Friedel–Crafts type reaction in the refluxing solvents of toluene and PPA thus forming the desired benzofuran **10** or benzothiophene **10'** as major isomers[19]. Following a Buchwald reaction and reductive amination, **10** or **10'** were assembled to give a substituted aniline **12** or **12'**, which was hydrolyzed and then coupled with pyridonyl methyl amine **13**[20] to finish the synthesis of **2** or **3**.

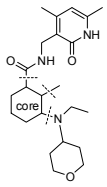


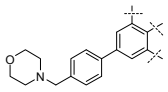
[a] Reagents and Conditions: (a) NBS, H_2SO_4, 60°C; (b) H_2SO_4, MeOH, reflux; (c) Fe powder, NH_4Cl, EtOH/H_2O, 70°C; (d) For X=O: $NaNO_2$, 20% H_2SO_4 ; For X=S: $NaNO_2$, HCl, Potassium ethyl xanthate, 70°C; KOH, MeOH, 60°C; (e) For X=O: 2-bromo-1,1-diethoxyethane, K_2CO_3, DMF, 70°C; For X=S: an addtional esterifiation was needed, CH_3I, K_2CO_3, DMF; (f) PPA, toluene, reflux; (g) Tetrahydro-2*H*-pyran-4-amine, $Pd_2(dba)_3$, BINAP, Cs_2CO_3, toluene, 100-110°C; (h) CH_3CHO, HOAC, $NaBH_3CN$, MeOH; (i) NaOH, THF/H_2O, 60°C; (h) **13**, EDCI, HOBT, Et_3N, DMF.

Scheme 1. Synthesis of compound **2/3**[a]

Remarkably, compound **2** and **3** showed comparable potency to EPZ-6438 in the EZH2 biochemical assay with IC_{50} values in the low nanomolar range. However, benzothiophene **3** showed only a weak potency in inhibiting the growth of Pfeffier cells, suggesting a further structure modification would be necessary to achieve sufficient potency, as measured by cell-based models. Benzofuran **2** gave the best activity with an IC_{50} of 240 nM against Pfeffier cell line. These results are summarized in **Table 1**.

Table 1 Preliminary SAR results with alternative scaffolds

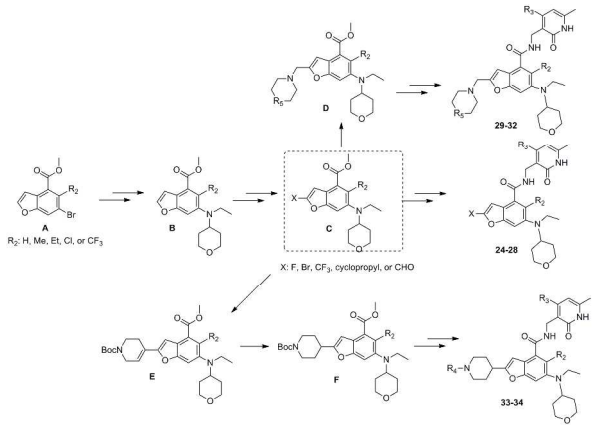


Compd	Structure	A677G[a] IC_{50} (nM)	Pfeffier[a] IC_{50} (nM)
EPZ-6438		4.0	38
1		20	n.d[b]
2		4.0	240
3		6.0	1830

[a]Data represent mean value of at least two experiments. [b]n.d. = not determined.

Based on the above preliminary results, we decided to focus on the benzofuran series to improve *in vitro* activity and drug-like properties of compound **2**. The following strategies were considered for improving the pharmaceutical profiles of our lead compound **2**. To decrease the metabolic issues caused by the pyridone fragment, we made a number of analogues with different substituents of the methyl group on the pyridone ring. Additionally, because the 2- or 3-position on the benzofuran ring might be another metabolic soft spot, we synthesized alternative 2- or 3-substituted benzofuran analogs to avoid potential metabolic issues. It was reported that the methyl group of the phenyl ring plays a critical role and is able to boost activity 10-fold according to Epizyme's pivotal study[8]. Based on this observation there was a potential that other groups could have a similar effect. Therefore, the previous findings were used to make a series of EZH2 inhibitors with the goal being increased potency and bioavailability.

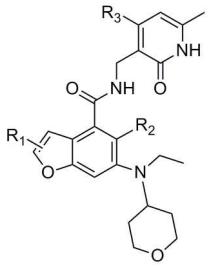
The general synthetic routes for those inhibitors are summarized in **Scheme 2**. Intermediate **A** was converted to a substituted aniline **B** via Buchwald coupling and reductive amination. Subsequently, intermediate **B** went through similar steps as compound **2** to obtain compounds **14-16**. In an alternative approach, intermediate **B** was efficiently functionalized to give intermediate **C,** which bore different X including F, Br, CF_3, Methyl, cyclopropyl, and carbonyl *etc*. Compounds **24-28** were synthesized via the previously described steps from compound **C**. If X was a carbonyl group, **C** was transformed to intermediate **D** via reductive amination, followed by routine steps to give compounds **29-32**. Intermediate **E** was synthesized through Suzuki coupling from **C** with a bromide substitution. Further selective hydrogenation reduced one of the double bonds to give intermediate **F**, followed by a series of routine reactions to obtain final products **33-34.**



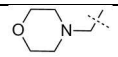
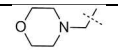
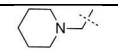
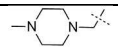
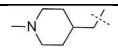
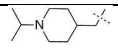
Scheme 2. Synthetic summary of other analogs

The SAR results are shown in **Table 2**. By switching R_3 from methyl to methoxyl, compound **14** exhibited not only similar enzymatic activity as **2**, but also showed much improved (>11-fold increase) cellular potency with an IC_{50} value of 20nM. However, other substitutions, such as ethyl or triflurometh**y**l, did not show improvement for compounds **15** or **16**. Subsequently, some alternative benzofuran analogs were evaluated. The substitution of the 2-methyl on compound **17** or **18** demonstrated better activity than the original compound **2**. In contrast, the 3-methyl substitution of compound **19** only showed weak potency with an IC_{50} of 1.3μM. These results suggested that the 2-position, but not the 3-position, on the benzofuran had the potential for optimization. Interestingly, the effect of methoxyl substitution was not observed as was previously seen with 2-substituted benzofuran analogs (14nM vs 21nM for IC_{50}). We then screened different R_2 at the phenyl ring in which a chloro substituted compound **23** kept a comparable potency of 150nM, but the triflurom**e**thyl substituted compound **22** and nonsubstituted compound **21** lost much of their potency in both biochemical and cellular assays. However, ethyl substituted compound **20**'s cellular potency was dramatically improved to 15nM compared to **2,** which bore the methyl substitution. To explore the SAR of the 2-position on the benzofuran, we first examined simple substitutes including F, Cl, CN, CF_3, and cyclopropyl. Compound **25**, **26**, **27,** and **28** exhibited excellent enzymatic (with IC_{50} less than 1nM) and cellular activity (with IC_{50} less than 20nM) with the exception of **24** which only had modest cellular activity. With the creation of these analogs, we further tested the compounds liver microsomal stability (Human/ Rat). However, most of the compounds were unstable, with $T_{1/2}$ less than 10 mins. To avoid metabolic issues, we rationalized that the assembly of classical drug-like fragments such as morphlinyl, piperidinyl, and piperazinyl at 2-positioned benzofuran ring might be beneficial. Additionally, these modifications have the potential of helping to increase the compound's solubility. Compounds **29-32,** with one CH_2 linker attached to a saturated heterocycle, exhibited better liver microsomal stability in addition to potent cellular activity. Furthermore, the more rigid compounds **33-34** gave superior liver microsomal stability (>0.5h). Especially the $T_{1/2}$ of compound **33** which was more than 1h, Compound **34** showed enzymatic activity and cellular activity of 4.0nM and 6.0nM, respectively. Based on the above data, compounds **33** and **34** became our candidates for *in vivo* study.

Table 2 Selected SAR of benzofuran series



Compd	R_1	R_2	R_3	A677G[a] IC_{50}(nM)	Pfeffier[a] IC_{50}(nM)
14	None	Me	MeO	4.0	20
15	None	Me	Ethyl	0.6	220
16	None	Me	CF_3-	4.6	3300
17	2-Me	Me	MeO	2.9	14
18	2-Me	Me	Me	3.8	21
19	3-Me	Me	MeO	5.9	1300
20	None	Ethyl	MeO	3.3	15
21	None	H	MeO	331	>5000
22	None	CF_3-	MeO	2000	n.d[b]
23	None	Cl-	None	6.5	150
24	2-F	Me	MeO	4.1	160

25	2-Cl	Me	MeO	0.4	21
26	2-CN	Me	MeO	0.8	17
27	2-CF_3	Me	MeO	2.7	17
28	2-cyclopropyl	Me	MeO	0.5	11
29		Ethyl	MeO	7.8	3.0
30		Ethyl	Me	4.6	14
31		Ethyl	MeO	6.4	2.0
32		Ethyl	MeO	0.1	8.0
33		Ethyl	Me	6.1	7.0
34		Ethyl	Me	4.0	6.0

[a]Data represent mean value of at least two experiments; [b]n.d= not determined

Compound **34**'s pharmacokinetic profile is summarized in **Table 3.** For i.v. administration, compound **34**'s clearance was modest with CLz/F of 26ml/min/kg and 32ml/min/kg in rats and mice, respectively. After a single 5 mg/kg and 10mg/kg oral dose of a CMC-Na suspension of **34** to rats and mice, its $AUC_{0\text{-}t}$ reached 239 ng/ml*h and 774 ng/ml*h with oral bioavailability as 9% and 16%, respectively. However, compound **33**'s, mice PK was inferior with $AUC_{0\text{-}t}$ 439ng/ml*h (data not shown)[21]. It was noted that **34**'s human, rat, and mouse plasma protein binding was 93.9%, 94.0% and 92.7% respectively, which implied compound **34** had excellent free drug proportion in the plasma across species.

Table 3 PK parameters of **34** (**EBI-2511**) in rats and mice

Pharmacokinetics[a] Parameters	Rats[a]		Mice[b]	
	p.o.	i.v.	p.o.	i.v.
Cmax(ng/ml)	93		257	
$AUC_{0\text{-}t}$ (ng/ml*h)	239	325	774	483
$T_{1/2}$ (h)	1.0		1.3	
CLz/F(ml/min/kg)	287	26	202	32
Vz/F(ml/kg)	24064	2556	21928	3679
MRT $_{0\text{-}\infty}$ (h)	3.0	1.0	2.7	1.3
Bioavailability (F)	9%		16%	

[a]Rats were administrated with dosages of 5mg/kg p.o. and 0.5mg/kg i.v., respectively. [b]Mice were administrated with dosages of 10mg/kg p.o. and 1.0mg/kg i.v., respectively.

In order to further assess the anti-proliferative effects by compound **34**, H3K27me3 western blot studies of compound **34** and EPZ-6438 were conducted as shown in **Figure 3**. The effect of EZH2 inhibition on H3K27 trimethylation (H3K27me3) in a cell-based setting was determined in Pfeiffer cells. Compound **34** significantly reduced cellular H3K27me3 levels in a dose-dependent manner with an approximate IC_{50} of 8nM, which was 3-fold more potent than EPZ-6438 (**Figure 3**). In addition to Pfeffier cell line, Compound **34** was shown active with IC_{50} value of 55 nM against WSU-DLCL2.

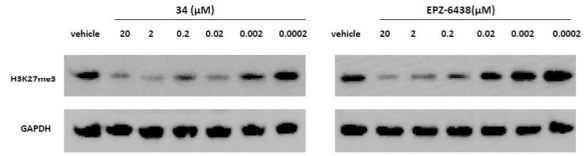


Figure 3 H3K27 trimethylation inhibition in Pfeiffer cells for **34** (**EBI-2511**) and EPZ-6438

The *in vivo* efficacy of compound **34** (**EBI-2511**) was evaluated in a Pfeiffer Xenograft mouse model as shown in **Figure 4**. Tumors were allowed to grow to a predetermined size (c.a. 150-200 mm^3) before administration of the testing compounds. **EBI-2511** was administered orally at, 10, 30, or 100 mg/kg once daily for 20 days, with EPZ-6438 serving as the reference compound (100mg/kg). As showed in **Figure 4**, **EBI-2511** displayed a dose-dependent inhibition on the tumor growth, resulting in 28% (10mg/kg), 83% (30mg/kg), and 97% (100mg/kg) reduction in tumor size. At the same dosage level, **EBI-2511** showed a superior anti-tumor efficacy to EPZ-6438 ($P<0.01$). It was noteworthy that no significant changes in body weights of all treatment groups were observed.

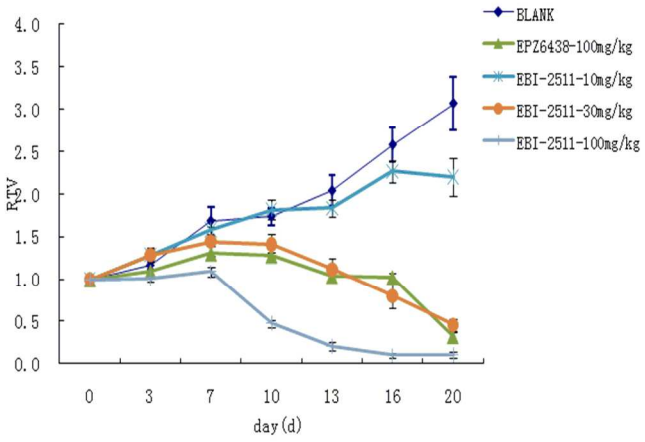


Figure 4. Pfeiffer Xenograft mice model study

In summary, we have discovered a novel series of EZH2 inhibitors with a benzofuran core via scaffold hopping based on EPZ-6438. Further rational optimization resulted in the development of the SAR series. One of the optimized analogs **EBI-2511** (**34**) demonstrated both excellent potency *in vitro* and superior efficacy *in vivo*, which implied that it might be possible **for EBI-2511** to achieve similar efficacy as EPZ-6438 but at lower doses in clinical studies. Further development of this molecule are in progress

ASSOCIATED CONTENT

Supporting Information

Experimental details for the synthesis and characterization of key intermediates and selected compound **2**, **3**, and **34** (**EBI-2511**); biochemical, cellular and H3K27 trimethylation

assays; Pharmacokinetics method; *in vivo* animal model. The Supporting Information is available free of charge on the ACS Publications website at: http://pubs.acs.org.

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Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

Notes

The authors declare no competing financial interest..

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