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Hybrid pyrimidine alkynyls inhibit the clinically resistance related Bcr-Abl^{T315I} mutant

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ABSTRACT

A series of pyrimidine alkynyl derivatives were designed and synthesized as new Bcr-Abl inhibitors by hybriding the structural moieties from GNF-7, ponatinib and nilotinib. One of the most potent compounds **4e** strongly suppresses Bcr-Abl^{WT} and Bcr-Abl^{T315I} kinase with IC_{50} values of 5.0 and 9.0 nM, and inhibits the proliferation of K562 and murine Ba/F3 cells ectopically expressing Bcr-Abl^{T315I} cells with IC_{50} values of 2 and 50 nM, respectively. It also displays good pharmacokinetics properties with an oral bioavailability of 35.3% and $T_{1/2}$ value of 48.7 h, and demonstrates significantly suppression on tumor growth in xenografted mice of K562 and Ba/F3 cells expressing Bcr-Abl^{T315I}. These inhibitors may serve as lead compounds for further developing new anticancer drugs overcoming the clinically acquired resistance against current Bcr-Abl inhibitors.

Chronic myeloid leukemia (CML) is a hematological malignancy representing about 20% of adult leukemia and characterized by the occurrence of the Philadelphia (Ph) chromosome. The first generation Bcr-Abl inhibitor imatinib has shown significant clinical benefit and become the first-line treatment of CML.[1,2] However, many patients eventually develop acquired resistance to imatinib. The 2-year incidence of resistance reaches 80% in the blastic phase, 40–50% in the accelerated phase, and 8–10% in the chronic phase.[3] Point mutation in the kinase domain of Bcr-Abl is the primary mechanism to imatinib resistance, and about 100 point mutations have been identified to date.[4–6] Nilotinib (**1**),[7,8] dasatinib[9,10] and bosutinib[11] have been approved for the treatment of adults in all phases of CML with resistance to the first Bcr-Abl inhibitor drug, whereas bafetinib[12] has also been developed in phase II clinical trials. However, these second-generation inhibitors are not capable of inhibiting all of the resistant mutants, especially the most notable Bcr-Abl^{T315I} gatekeeper mutation accounting for 15–20% in all clinical acquired resistance.[13] Thus, T315I mutation induced resistance remains a serious medical problem.

Structurally, the T315I mutation diminished a key hydrogen bond interaction between the inhibitors and Thr315 residue in the kinase domain of Abl protein. The bulky isoleucine side chain also makes a steric clash to prevent inhibitor binding into the hydrophobic pocket.[14,15] Several third generation inhibitors are capable of inhibiting the Bcr-Abl^{T315I} mutant have been identified.[16,17] Examples include the type I inhibitors PPY-A,[18] SGX-393,[19,20] c-Src/Abl dual inhibitors TG100598 and TG101223,[21,22] and aurora inhibitors MK-0457,[23,24] PHA-739358,[25,26] and AT9283,[27,28] as well as non-ATP competitive or allosteric inhibitors ON012380[29] and DCC2036.[30] However, these molecules have to be formulated for intravenous administration, and clinical development for MK-0457 has been discontinued due to cardiac toxicity.[24,31]

Recently, the 'third-generation' type II Bcr-Abl inhibitors GZD824,[32] GNF-7 (**2**),[33] AP24534 (**3**)[34,35] and 5-(arenethynyl)hetero-monocyclic derivatives[36] were reported to strongly inhibit Bcr-Abl^{T315I} and other mutants. AP24534 (ponatinib, Iclusig®) has been approved for the treatment of resistant or intolerant CML and Ph$^+$ ALL patients against imatinib, especially those harboring Bcr-Abl (T315I) mutation.[35,37] However, US FDA soon temporarily suspended the marketing of this drug due to the increasing numbers of blood clots observed in ponatinib-treated patients.[37] The drug was later reauthorized for sale after a revised indication statement and a boxed warning were made by the manufacture. As acquired resistance increasingly observed in the clinic, the newly

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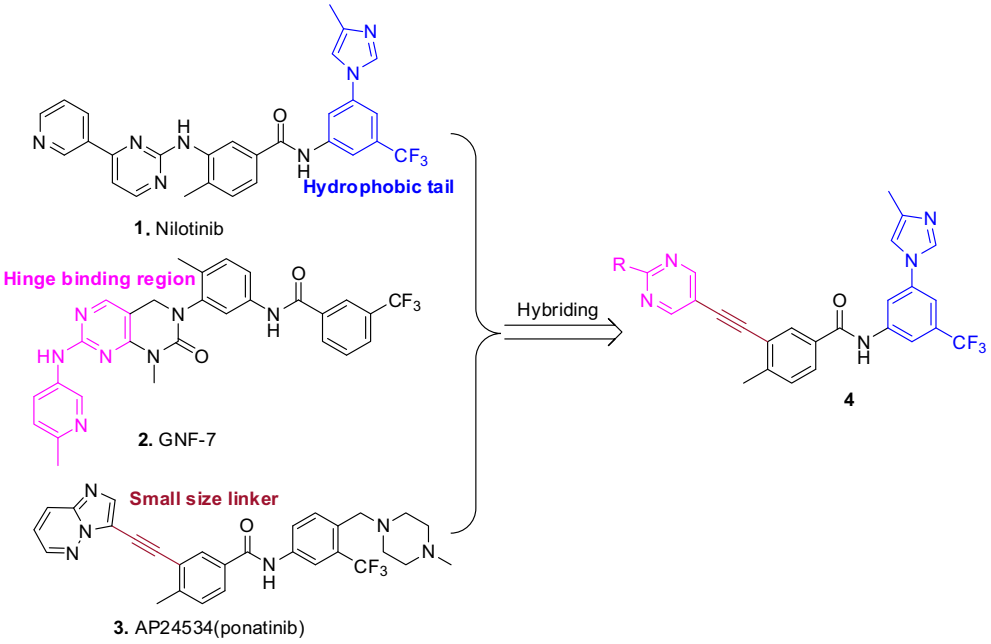


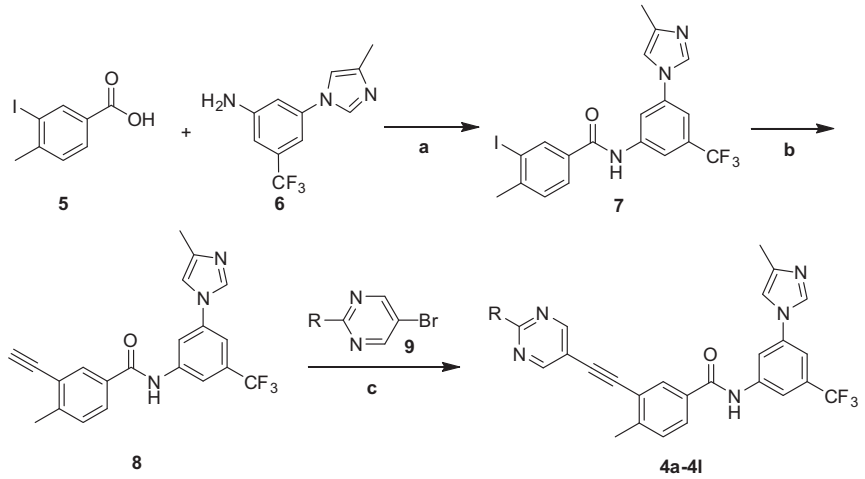
Figure 1. Design of new Bcr-Abl inhibitors by hybriding the chemical structures of nilotinib, GNF-7 and AP24534.

discovered Bcr-Abl inhibitors are required to not only suppress T315I mutant but also display reduced side effects.

Structural feature analysis reveals that almost all of the type-II Bcr-Abl inhibitors contain three crucial chemical elements: a kinase hinge binding region, a hydrophobic group binding to the back pocket of protein and a suitable linker between these two moieties. Based on the pharmacophore investigation, a series of *N*-(3-(4-methyl-1*H*-imidazol-1-yl)-5-(trifluoromethyl)phenyl)-3-(2-(pyrimidin-5-yl)ethynyl) benzamides (**4a**–**4l**) were designed and synthesized as new potential Bcr-Abl inhibitors by hybriding the key elements from GNF-7, ponatinib and nilotinib (Fig. 1). In the new inhibitors, a substituted pyrimidine is used as a hinge binding group, the 3-(4-methyl-1*H*-imidazol-1-yl)-5-(trifluoromethyl)phenyl hydrophobic moiety is 'borrowed' from the corresponding design in nilotinib. A small triple bond linker is also introduced by following AP24534 to avoid the potential steric clash with I315 in the resistant mutant.

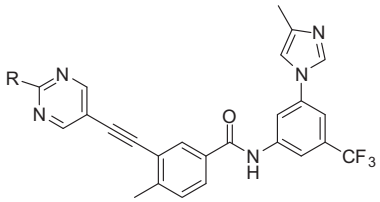
The synthesis of compounds **4a**–**4l** is outlined in Scheme 1. The Sonogashira coupling was used as the key reaction in the synthetic route.[38] Briefly, the iodo-intermediate **7** was afforded by amide condensation of 3-(4-methyl-1*H*-imidazol-1-yl)-5-(trifluoromethyl)aniline (**6**) with freshly prepared 3-iodo-4-methylbenzoyl chloride starting with 3-iodo-4-methylbenzoic acid (**5**). Treatment of **7** under palladium catalysis afforded the Sonogashira coupling products, which was further deprotected to produce the alkyne **8**. The desired products **4a**–**4l** were obtained by using another Sonogashira coupling of **8** with substituted 3-bromopyrimidine (**9**).

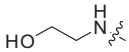
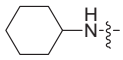
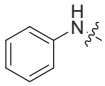
Kinase inhibitory activities of the designed compounds **4a**–**4l** against Bcr-Abl^{WT} and Bcr-Abl^{T315I} were evaluated by using a well established FRET-based Z'-Lyte assay.[39] FDA-approved drugs imatinib, nilotinib, dasatinib and ponatinib were used as positive controls to validate the screening conditions. As shown in Table 1, imatinib, nilotinib and dasatinib potently inhibit Bcr-Abl^{WT}, but



Scheme 1. Reagents and conditions: (a) i. $SOCl_2$, reflux; ii. THF, Et_3N, 0 °C–rt; 90%, (b) i. $Pd(dppf)_2Cl_2$, CuI, DIPEA, DMF, 80 °C; ii. K_2CO_3, MeOH, rt, 90%; (c) $Pd(OAc)_2$, PCy_3, CuI, DIPEA, DMF, 80 °C, 60–80%.

Table 1
Bioactivities of new hybrid Bcr-Abl inhibitors



Compd	R	Kinase inhibition (IC_{50}, μM)		Cell inhibition (IC_{50}, μM)		
		Bcr-Abl^{WT}	Bcr-Abl^{T315I}	K562	Ba/F3 ^{WT}	Ba/F3^{T315I}
4a	NH_2-	0.008	0.007	0.001	0.002	0.078
4b		0.010	0.006	0.007	0.003	0.085
4c		0.030	0.019	0.006	0.008	0.113
4d		0.125	0.187	0.027	0.023	1.158
4e		0.005	0.009	0.002	0.002	0.050
4f		0.018	0.019	0.013	0.017	1.077
4g		0.093	0.458	0.030	0.049	2.711
4h		0.028	0.024	0.023	0.038	0.135
4i		0.238	0.161	0.111	0.208	2.396
4j		0.904	>1	0.640	0.683	>10
4k		>1	>1	>0.925	>1	>10
4l		0.3	>1	0.355	0.292	8.433
Imatinib	–	0.399	>1	0.280	–	>1
Nilotinib	–	0.014	>1	0.022	–	>1
Dasatinib	–	0.003	>1	0.001	–	>1
AP24534	–	0.001	0.0009	0.003	–	0.006
GNF-7[a]	–	0.133	0.061	<0.005	–	0.011

[a] Reported activity data.[33]

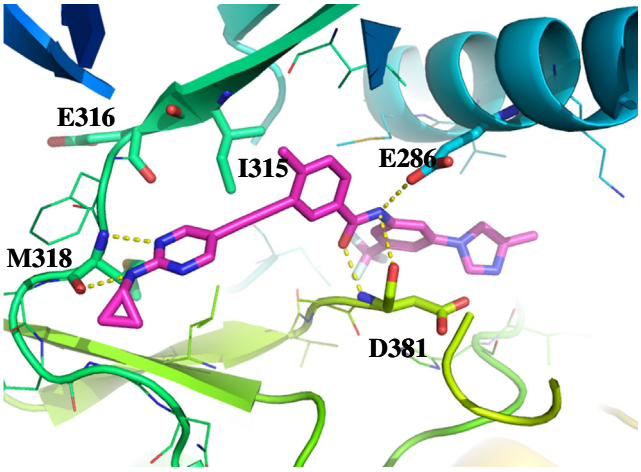


Figure 2. The predicted binding mode of **4e** with Bcr-Abl^{T315I}. Hydrogen bonds are indicated by yellow hatched lines to key amino acids.

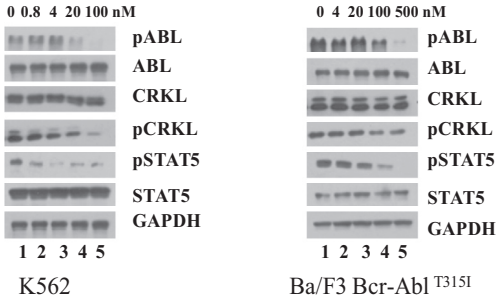


Figure 3. Compound **4e** inhibits Bcr-Abl signaling in K562 and Ba/F3 stable cells expressing Bcr-Abl^{T315I}. K562 is a human CML cell with positive Bcr-Abl^{WT}. Cells were treated with **4e** at the indicated concentrations for 4.0 h, and whole cell lysates were then subjected to Western blot analyses. The results are from three independent experiments.

they are all inactive against Bcr-Abl^{T315I}. However, ponatinib suppresses the enzymatic activity of wild-type and the T315I mutant with low nanomolar IC_{50} values. Similar to the reported data, GNF-7 also potently suppresses the enzymatic activity of T315I mutation with IC_{50} values of 61 nM.[33]

Under the experimental conditions, the first hybrid compound **4a** with a free amino group at R position inhibits the kinase

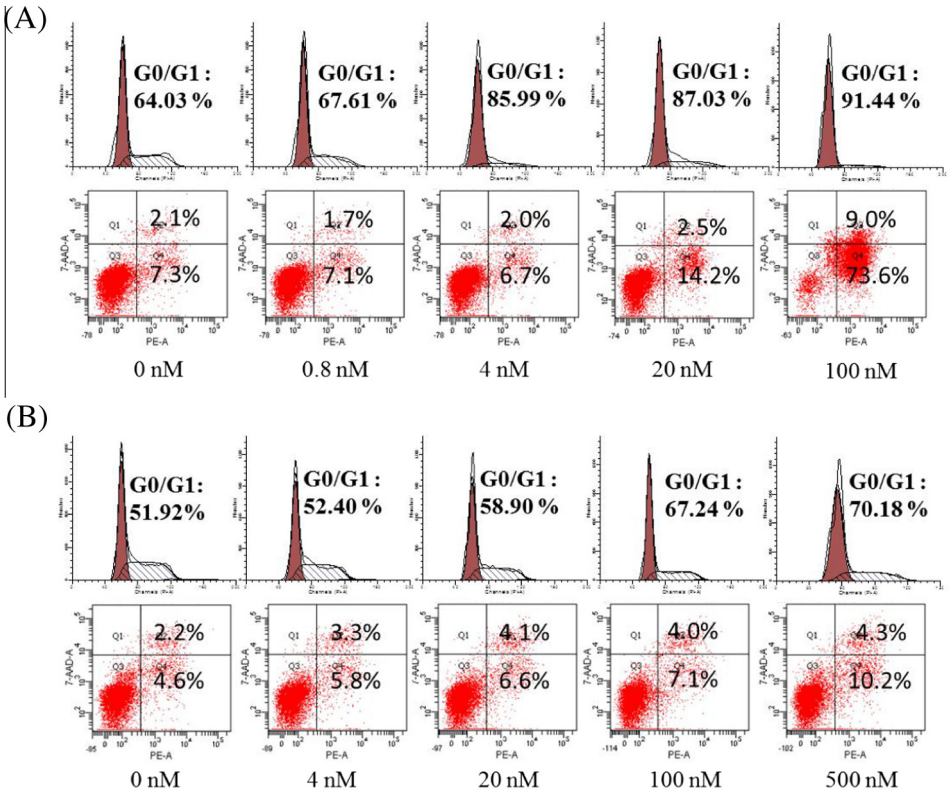


Figure 4. (A) Effect of **4e** on cell cycling and apoptosis in K562 cells. (B) Effects of **4e** on cell cycling and apoptosis in Ba/F3-T315I cells. K562 cells and Ba/F3-T315I cells were both exposed to **4e** at the indicated concentrations for 24 h, then cells were fixed and analyzed by flow cytometry. Data represent the mean ± 95% CI of three independent experiments.

Table 2
The pharmacokinetic profiles of **4b** and **4e** in rats

Compd	Pharmacokinetics parameters						
	Route	Dose (mg/kg)	$AUC_{0-\infty}$ (μg/L * h)	C_{max} (μg/L)	T_{max} (h)	$T_{1/2}$ (h)	*F* (%)
4e	Oral	25	251684	6205	4.5	48.7	35.3
	Iv	10	285187	49750	0.033	71.7	
4b	Oral	25	48574	3090	2	7.8	27.1
	Iv	10	35809	10975	0.033	7.5	

activities of both Bcr-Abl^{WT} and Bcr-Abl^{T315I} with IC_{50} values of 0.008 and 0.007 μM, respectively, which is almost comparable to that of ponatinib and about 10-times more potent that GNF-7 (Table 1). Further structural optimization studies reveal that the substituent at *R*-position significantly affect the kinase inhibitory activities against Bcr-Abl^{WT} and Bcr-Abl^{T315I}. Although the methyl amino analogue **4b** displays identical potency to that of **4a**, the ethyl (**4c**), iso-propyl (**4d**), 2-hydroxyethyl (**4f**), cyclo-hexanyl (**4g**), tert-butyl (**4h**) and 4-phenyl (**4i**) substituted compounds are significantly less potent than **4a**. The IC_{50} values are 0.30 and 0.19, 0.125 and 0.187, 0.018 and 0.019, 0.093 and 0.458, 0.028 and 0.024, 0.238 and 0.161 μM, respectively, against Bcr-Abl^{WT} and Bcr-Abl^{T315I}. *N*,*N*-disubstituted moieties such as piperidinyl (**4j**), pyrrolidin-1-yl (**4k**) and morpholino (**4l**) groups are all detrimental to the inhibitory activities against both wild-type Bcr-Abl and the resistant mutant. The resulting compounds almost totally abolish their inhibitory effect against Bcr-Abl^{T315I} kinase (IC_{50} >1.0 μM). Finally, we are pleased to find the cyclopropyl substituted compound **4e** is as potent as to compound **4a** but is with improved solubility, which inhibits Bcr-Abl^{WT} and Bcr-Abl^{T315I} with IC_{50} values of 5.0 and 9.0 nM, respectively.

Antiproliferative activities of the new hybrids are also examined against Bcr-Abl positive K562, and murine Ba/F3 cells ectopically expressing Bcr-Abl^{WT} and Bcr-Abl^{T315I} (termed as Ba/F3 WT and Ba/F3 T315I cells). The results were summarized in Table 1. Consistent with their kinase inhibitory activities, most of the compounds show promising anti-proliferative activities. For instance, the most promising compound **4e** strongly inhibits the proliferation of K562, Ba/F3 WT and Ba/F3 T315I cells with IC_{50} values of 2, 2 and 50 nM, respectively, which is similar to that of GNF-7.

A molecular docking study was also performed to get insight investigation on the binding mode of compound **4e** with Bcr-Abl^{T315I} (PDB code: 3IK3) by using Glide module (Glide, version 5.7 Schrödinger, LLC, New York) with standard precision scoring function. Compound **4e** binds to the ATP binding site of Bcr-Abl^{T315I} with a DFG-out conformation (Fig. 2), which is similar to that of ponatinib.[35,37] The *N*-cyclopropyl amino pyrimidinyl motif of **4e** forms two essential hydrogen bonds with the backbone of Met318 in the hinge region of Bcr-Abl^{T315I}. This observation highlights the great contribution of a hydrogen-donating NH moiety to the kinase inhibitory activity, which has been confirmed by varying the substituent in *R*-position (i.e., compounds **4j**, **4k** and

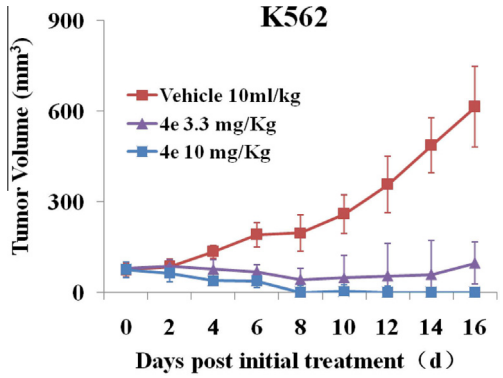


Figure 5. Compound **4e** potently suppresses tumor growth in K562 xenograft model of human CML. Mice bearing K562 xenografts were dosed orally once a day with **4e** at 3.3 and 10 mg/kg dosages for 16 consecutive days. The data are representative of two independent experiments.

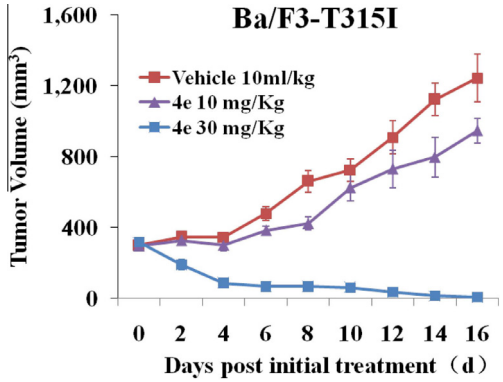


Figure 6. Compound **4e** potently suppressed tumor growth in allografted models of Ba/F3 cells expressing Bcr-Abl^{T315I}. Mice bearing allografted Ba/F3 Bcr-Abl^{T315I} cells were dosed orally once a day with **4e** at 10 mg/kg and 30 mg/kg dosages for 16 consecutive days. The data are representative of two independent experiments.

4l). The amide forms three hydrogen bonds with Glu286 and Asp381, and the trifluoromethylphenyl group binds deeply into a hydrophobic back pocket. The alkynyl linker made favorable van der Waals interactions with the gatekeeper Ile315 avoiding steric clash.

Taking **4e** as an example, the kinase inhibition of the new inhibitors is further validated by investigating their suppressing effect on the activation of Bcr-Abl and its downstream signals in cells harboring different status of Bcr-Abl kinase. As shown in Figures 3 and 4e dose-dependently inhibits the phosphorylation of Bcr-Abl in both K562 and Ba/F3 T315I cells. The phosphorylation level of the downstream signal proteins such as CRKL and STAT5 were also obviously decreased, while the total protein levels of Bcr-Abl, CRKL, STAT5 and GAPDH remained unchanged. The data further support strong target inhibition of the newly designed compounds. Not surprisingly, the three FDA-approved drugs (imatinib, nilotinib and dasatinib) demonstrated no obvious suppression of Bcr-Abl^{T315I} activation.[32]

Further flow cytometric analysis revealed that compound **4e** also significantly induced the G0/G1 phase arrest and apoptosis in a dosage dependent manner in both K562 (Fig. 4A) and Ba/F3-T315I cells (Fig. 4B), respectively, which is a consequent response of Bcr-Abl ^{WT} and Bcr-Abl ^{T315I} kinase inhibition.

Two potent inhibitors (**4b** and **4e**) are continually evaluated for their pharmacokinetic (PK) properties in rats. In general, **4e** bearing *N*-cyclopropyl demonstrates higher AUC and C_{max} than the *N*-methyl compound **4b** (Table 2). For instance, **4e** has a bioavailability of 35.3% and a half-life of 48.7 h. It is also noteworthy that the C_{max} of compound **4e** reaches 6205 μg/L (about 12 μM) after a 25 mg/kg oral administration, which is about 1333 folds of its IC_{50} value against Bcr-Abl ^{T315I}, indicating the potential in vivo efficacy in animal models.

Giving its promising kinase activity and PK properties, compound **4e** is further evaluated in subcutaneous K562 xenograft model of *human* CML. The animals were orally dosed with **4e** at 3.3 and 10 mg/kg/day. As shown in Figures 5 and 4e dose-dependently inhibits the growth of the K562 tumor xenograft when administered once daily for 16 consecutive days via oral gavage and almost completely eradicates the tumor at doses of 10 mg/kg/day after 8 days of treatment. Notably, after the cessation of treatment (16 days of dosing), there was no sign of tumor recurrence in the following 7 days. The compound **4e** is well tolerated with no mortality or significant body loss (<5% relative to vehicle-matched controls) during the treatments.

Compound **4e** also demonstrates promising antitumor effect in an allograft model of Ba/F3 T315I cells. The animals were administered **4e** at doses of 10 and 30 mg/kg via oral gavarge once daily for 16 days. As shown in Figure 6, compound **4e** dose-dependently inhibits tumor growth in the allograft models of Ba/F3 T315I cells. Although compound **4e** does not show obvious inhibition of tumor growth at dose of 10 mg/kg/day, it induces almost complete tumor regression at a dose of 30 mg/kg/day after a 16 days consecutive treatment.

In summary, a series of pyrimidine alkynyl derivatives are designed and synthesized as new Bcr-Abl^{T315I} inhibitors by hybriding the structural moieties from GNF-7, ponatinib and nilotinib. The resulting compounds strongly suppressed the activities of Bcr-Abl^{WT} and Bcr-Abl^{T315I} kinases and potently inhibited the proliferation of K562 and Ba/F3 T315I cells. The most promising compound **4e** displays comparable potency with that of ponatinib and GNF-7 in cancer cell antiproliferative assay. Compound **4e** also dose-dependently induces the G0/G1phase arrest and apoptosis of K562 and Ba/F3 T315I cells. It also displays good pharmacokinetics properties and potently suppresses tumor growth in xenografted mice bearing K562 and Ba/F3 cells expressing Bcr-Abl^{WT} and Bcr-Abl^{T315I} in vivo assay. These inhibitors might serve as lead compounds for further developing new anticancer drugs.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmcl.2015.07.006.

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