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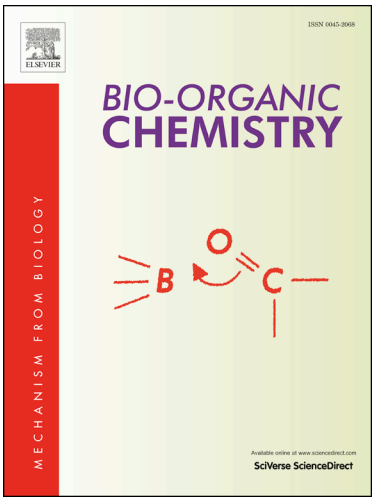

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Design, Synthesis and Anti Leukemia Cells Proliferation Activities of Pyrimidylaminoquinoline Derivatives as DOT1L inhibitors

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ABSTRACT

A series of novel pyrimidylaminoquinoline derivatives **8(a-i)** and **9(a-i)** containing amino side chain, and the bisaminoquinoline analogs **3(b-e)** have been designed and synthesized by structural modifications on a lead DOT1L inhibitor, **3a**. All the compounds have been evaluated for their DOT1L inhibitory activities. The results showed that most of the compounds have strong anti DOT1L activities. Compounds **3e**, **8h** and **9e** are the most potential ones from each category with the IC_{50} values of 1.06 ± 0.35 μM, 5.72 ±1.56 μM and 3.55±1.28 μM, respectively. Such inhibitors expressed significant binding interactions with DOT1L by surface plasmon resonance (SPR)-based binding assay. The results of molecular docking experiments suggested that they could occupy the SAM binding pocket of DOT1L. Compounds **8h** and **9e** exhibited better inhibitory activities but poor selectivities against the both MLL-rearranged MV4-11 cells and the non MLL-rearranged Kasumi-1 cells than those of **3a** and **3e**, which suggested that the introduction of the amino side chain would be beneficial for their anti leukemia cells proliferation activities, possibly due to the improvement of the fat solubility. Additionally, the direct cellular inhibition activities were found that compound **9e** could effectively down-regulate both the level of H3k79 methylation and MLL-rearranged leukemia gene expression of Hoxa9 and Meis1 in MV4-11 in the qRT-PCR and western blot studies. These observations suggested DOT1L was one of the potential targets but perhaps not the most pivotal one for these compounds, which made their poor selectivities against leukemia cells proliferation.

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1. Introduction

Disruptor of telomeric silence 1-like (DOT1L), a histone lysine methyltransferase (HKMTs), is responsible for specifically catalyzing the mono-, di-, and tri- methylation of the histone H3-lysine79 (H3K79) using (*S*)-adenosyl-L-methionine (SAM, Fig 1) as the methyl donor (enzyme cofactor).[1] The aberrant methylation of H3K79 mediated by DOT1L would induce and boost the occurrence and maintenance processes of mixed linage leukemia (MLL) by promoting the expression of leukemogenic genes, such as HOXA9 and MEIS1.[2] Thus, DOT1L inhibitors become the promising therapeutic tools to treat MLL due to the critical roles of DOT1L in leukemogonesis.[3]

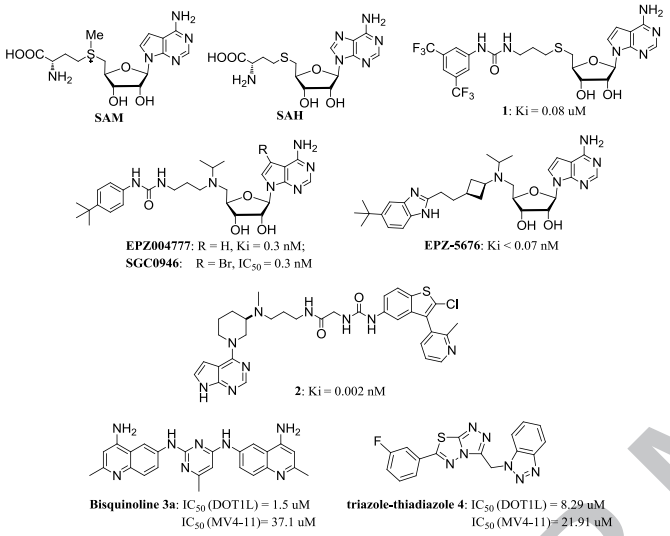
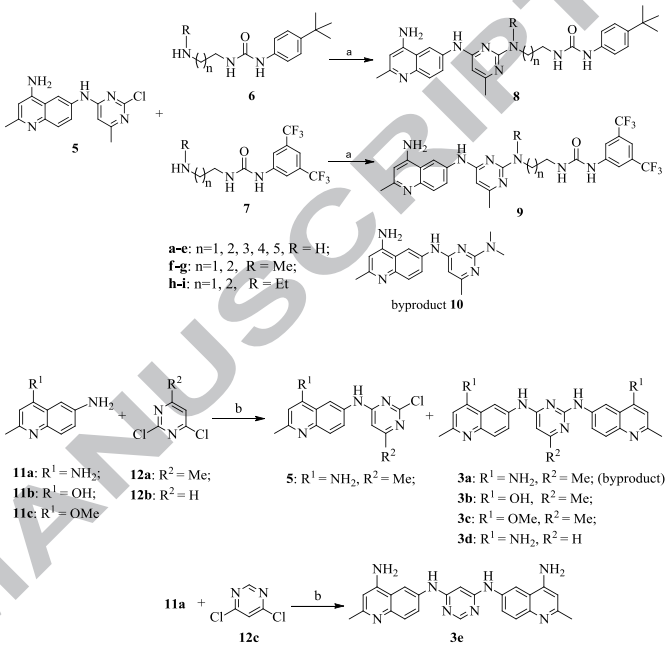


Fig.1 The structures of SAM, SAH, and DOT1L inhibitors.

DOT1L inhibitors have been divided into two kinds, nucleoside and nonnucleside inhibitors. The former is designed based on the structures of SAM and its corresponding enzymatic reaction product (*S*)-adenosyl-L-homocysteine (SAH).[4] Epizyme international company,[5] the Song laboratory,[6] and the Structural Genomics Contrsortium[7] have developed several dozen nucleoside inhibitors, among them, compound **1**, EPZ004777, SGC0946, and EPZ5676 (Fig 1) are the most effective and selective inhibitors of DOT1L. As an alternative way to inhibit DOT1L, the nonnucleside inhibitors, such as **2** (Fig 1), mainly target to the induced binding pocket adjacent to the SAM binding site, have been greatly developed by the Novartis.[8] Recently, the Luo laboratory[9] has identified **3a**, the bisaminoquinoline linked through 4-methyl pyrimidyl, as the first novel nonnucleside DOT1L inhibitor through structure-based virtual screening.[10] Subsequently, the same lab found inhibitor **4** (Fig 1) with triazolo-thiadizole scaffold by using target-specific scoring function.[11] Both inhibitors show high inhibitory activities towards DOT1L, but weak activities against MLL-rearranged cells (MV4-11) proliferation, perhaps due to their poor cell membrane permeability. Molecular docking analysis found that the inhibitors competitively occupies the binding site of SAM, different from the Novartis's ones.

As the putative binding modes in mind, we hypothesize that introducing the amino side chain to **3a** would increase its cell membrane permeability, and would be beneficial to improving its activity against leukemia cells. Herein, we would like to report the design and synthesis of the pyrimidylaminoquinoline compounds **8(a-i)** and **9(a-i)** bearing with the amino side chains by the privileged fragment linking of **5** and **6 (**or **7)**. Furthermore, the bisaminoquinoline analogs **3(b-e)** were also prepared to explore the chemistry space of the parent inhibitor **3a** to increase its structural diversity for structure activity relationships (SAR) analysis. All of the new compounds were evaluated for their DOT1L inhibitory activities and anti leukemia cells proliferation activities, including the MLL-rearranged MV4-11 cells and the non MLL-rearranged Kasumi-1 cells.



Scheme 1. The design and synthesis of compounds **8, 9** and **3(b-e)**. *Conditions and reagents*: (a) glycol, DIPEA, 110°C; (b) glycol, 12 N HCl, 50 °C.

2. Results and Discussions

2.1. Chemistry

Synthesis of compounds **8(a-i)**, **9(a-i)** and the bisamino quinoline analogs **3(a-e)** was outlined in Scheme 1. Briefly, the 4,6-diamino quinoline **11a** was prepared according to the literatures[12] and **11(b-c)** were obtained as the intermediates in the synthetic process of **11a** (in Supporting Information). The reaction of **11a** with 2,4-dicholo-6-methyl-pyrimidine **12a** in 12N HCl afforded the key intermediate **5**, accompanied by side reaction leading to the byproduct bisaminoquinoline **3a**. Then, following the same procedure, the bisquinoline analogs **3(b-e)** were synthesized by the treatment of **11(a-c)** with a variety of dicholopyrimidines **12(a-c)**, respectively. The various amino side chains **6** and **7** were achieved by the reaction of the commercially available diamines with the corresponding aryl isocyanates (in Supporting Information). The electrophilic substitutions of **5** with **6** or **7** provided the final products **8(a-i)** and **9(a-i)**, respectively. The byproduct **10** was generated when DMF was used as the solvent.

The structures of all the newly compounds were determined by NMR (Fig S1) and (HR) MS. Both analytical and spectral data of compounds are in agreement with the proposed structures.

2.2. DOT1L inhibitory activity

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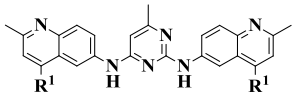
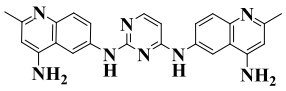
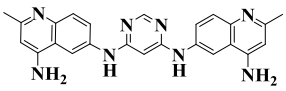
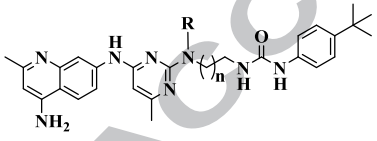
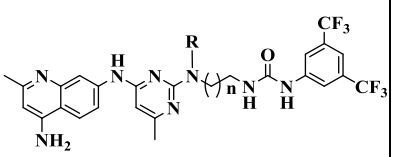
All the newly synthesized compounds **8(a-i)**, **9(a-i)**, **3(b-e)**, **5** and **10** were evaluated for their DOT1L inhibitory activities at the concentrations of 20 μM and 5 μM via a ^{3}H labeled radioactive methylation assay. The parent compound **3a** was used as the positive control. The results are shown in Table 1 and Fig S2. The results show that all of the tested compounds except **5** and **10** have good to potent DOT1L inhibitory activity in range of 38%-100% at 20 μM. Among them, compounds **3d** and **3e** have the strongest DOT1L inhibitory activities with near 100% inhibition at both concentrations, similar with those of **3a**. Subsequently, the compounds with the inhibition rate above 50% at 5 μM were chosen to further evaluate their IC_{50} values (Table 1, Fig S3). It could be seen that in serial of **3**, the bisaminoquinoline analogs **3d** and **3e** exhibited the most potential activities against DOT1L with the IC_{50} values of 1.08 ± 0.23 μM and 1.06 ± 0.35 μM, respectively. In serial of **8**, compounds **8b**, **8f**, and **8i** showed significant inhibition with the IC_{50} values lower than 10 μM, and **8h** was the most active one with the IC_{50} value of 5.72 ± 1.56 μM. In serial of **9**, **9e** with the IC_{50} value of 3.55 ± 1.28 μM was the most effective one, and **9b** had the good activity with the IC_{50} values lower than 10 μM. However, **5** and **10** exhibited weak activities against DOT1L. According to the structures and the inhibitory activities of the newly synthesized compounds, **3e**, **8h** and **9e**, the most potential one from each category, were chosen to be further validated.

Table 1. Structures, DOT1L inhibitory and anti the leukemia cells activities of **3a** and its derivatives.

	Cpd.	R/R[1]	n	anti-DOT1L			anti-MV4-11[c]	anti-Kasumi-1[d]
				Inhibition ratio (20 μM, %)	Inhibition ratio (5 μM, %)	IC_{50} (mean±SD, μM)	IC_{50} (mean±SD, μM)	IC_{50} (mean±SD, μM)
	**3a**	NH_2	-	100	93.9	1.5[10]	37.1[10]	a
	3b	OH	-	65	53	4.35 ± 1.05	a	a
	3c	OMe	-	66	63	21.67 ± 2.08	a	a
	**3d**	-	-	100	100	1.08 ± 0.23	a	a
	**3e**	-	-	101	99	1.06 ± 0.35	30.54 ± 1.32	45.75 ± 3.14
	**8a**	H	1	51	27	a	a	a
	8b	H	2	85	71	7.79 ± 3.05	a	a
	8c	H	3	58	18	a	a	a
	8d	H	4	52	18	a	a	a
	8e	H	5	53	29	a	a	a
	8f	Me	1	84	53	6.04 ± 1.10	a	a
	8g	Me	2	90	48	a	a	a
	8h	Et	1	83	54	5.72 ± 1.56	1.62 ± 0.17	3.80 ± 0.60
	8i	Et	2	97	60	7.91 ± 1.32	a	a
	**9a**	H	1	66	36	a	a	a
	9b	H	2	71	54	8.77 ± 1.16	a	a
	9c	H	3	66	31	a	a	a
	9d	H	4	48	47	a	a	a
	9e	H	5	52	51	3.55 ± 1.28	1.29 ± 0.32	4.25 ± 1.04

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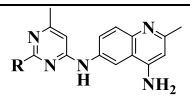
	9f	Me	1	49	48	a	a	a
	9g	Me	2	68	41	a	a	a
	9h	Et	1	38	12	a	a	a
	9i	Et	2	48	36	a	a	a
	**5**	Cl	-	b	b	a	a	a
	10		-	28	1	a	a	a

a: Not determined;

b: No activity;

c: MV4-11: MLL-rearranged cell;

d: Kasumi-1: a leukemia cell line without MLL-rearrangement.

2.3. Surface Plasmon Resonance (SPR)-Based binding assay

To further validate the binding of DOT1L and the potent inhibitors **3e**, **8h**, and **9e**, the SPR assays were conducted to determine their interactions. As shown in Fig 2, the tested compounds showed good interactions with DOT1L. The equilibrium dissociation constant (K_D) for **3e** and DOT1L was 0.80 μM, nearly the same with that of **3a**.[10] And the K_D for **8h**, **9e** and DOT1L were 3.52 μM and 1.31 μM, respectively. The values of their K_D are consistent with their anti-DOT1L activities, indicating that the inhibitors can bind to DOT1L and then inhibit DOT1L activity *in vitro*.

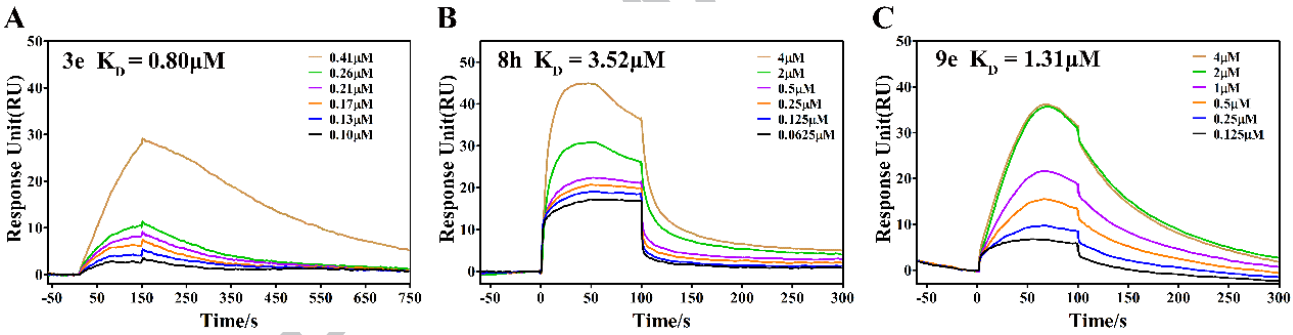


Fig.2 SPR assays of the binding between DOT1L and the inhibitors. The concentrations of the three compounds injected over the CM5 chip are indicated, and the SPR curves for these inhibitors binding with DOT1L are shown. (A) The value of K_D for **3e** binding with DOT1L was 0.80 μM. (B) The value of K_D for **8h** binding with DOT1L was 3.52 μM. (C) The value of K_D for **9e** binding with DOT1L was 1.31 μM.

2.4. Binding mode and Structure-Activity Relationships (SARs) analysis

In order to achieve some visual insights into the binding interactions, compound **3e** was docked into the DOT1L crystal structure (PDB ID 3QOW) in XP mode using Glide[13] module of maestro[14] to generate putative binding poses. Since compounds **8h** and **9e** have the structural similarities with EPZ004777 containing a urea side chain, the crystal structure of EPZ004777 in complex with DOT1L (PDB ID 4EKI) was used for the docking studies. As shown in Fig 3A and 3B, compounds **3e**, **8h**, and **9e** fitted in the pocket as well as **3a**, SAM and EPZ004777 did. In the predicted binding poses of the compounds (Fig 3C-3E, Fig S4), the pyrimidine ring moiety of **3e** forms Pi-Pi stacking interactions with residue F223, similar to that of **3a** and SAM. The three compounds form hydrogen bonds with V135, D161, G163, D222 and N241, respectively. Besides, some nonpolar interactions like hydrophobic interactions were also formed between DOT1L and the compounds. This was similar to that of compound **3a** or EPZ004777. Compared to EPZ004777, though the Pi-Pi stacking interactions between **8h**, **9e** and F223 were not as strong as that between F223 and EPZ004777, cation-Pi interactions were formed between positive charged **8h** and F245, and a salt bridge was formed between **9e** and E186. The inhibitory activities of **8h** and **9e** against DOT1L were slightly lower than those of **3e** and **3a** possibly due to their flexible side chains. In conclusion, the putative binding modes of the three compounds were in consonance with their inhibitory activities and our experimental data.

We further found some relationships between the compounds structures and their anti-DOT1L activities. The structure-activity relationships (SARs) were analyzed on the basis of the anti-DOT1L activities. In serial of **3**, the bisaminoquinoline analogs **3d** and **3e** exhibited the most potential activities against DOT1L, while the 4-NH_2 on quinoline ring was replaced with OH or

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OMe, such as **3b** and **3c**, the activities reduced markedly, especially in the case of **3c**. These results suggested that the 4-NH_2 on quinoline had an important role to maintain their DOT1L inhibitory activity, while the slight changes on the pyrimidine linker would make subtle influence. In serial of **8,** possessing the *t*-butyl phenyl at the end of the amino chain, **8b**, **8f**, **8h** and **8i** showed significant inhibition against DOT1L, which suggested that the short chain length (n = 1 or 2) was more favorable for their inhibitory activities, and the R group (H, Me, or Et) seemed to have little influence on the inhibition. In serial of **9**, fixed as the terminal 3,5-ditrifluoromethyl phenyl, **9e** with the long chain (n = 5, R = H) was the most active one, and **9b** (n = 2, R = H) had the good activity, implying that H atom (R group) might be better accommodated into the DOT1L binding site, while the big R substituents, such as methyl and ethyl groups, would be a negative effect on their anti-DOT1L activities. Furthermore, the introduction of Cl atom or *N*,*N*-dimethyl group to the pyrimidine instead of the amino side chain, such as compounds **5** and **10**, would have a detrimental effect or would lead to a markedly loss in the activity. Above insights should help to guide further design and synthesis of the more potent DOT1L inhibitors derived from compound **3a**.

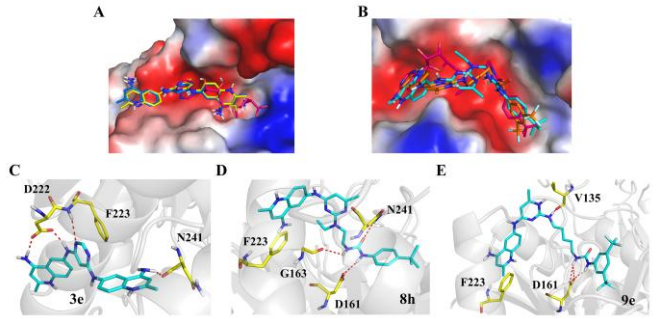

Fig.3 Putative binding mode of **3e**, **8h** and **9e** in SAM binding site of DOT1L. (A) SAM binding site of DOT1L (PDB ID 3QOW) occupied by **3e** aligned with **3a** and SAM. (B) SAM binding site of DOT1L (PDB ID 4EKI) occupied by **8h** and **9e** aligned with EPZ004777. **3a** is shown as yellow sticks, **3e** is shown as marine sticks, **8h** is shown as cyan sticks, **9e** is shown as tv_orange sticks, SAM and EPZ004777 are shown as hotpink sticks. The protein structures are shown in vacuum electrostatics. (C, D, E) Close-up view of the important interactions participated in stabilizing **3e, 8h** and **9e** in the SAM binding pocket. The protein structure is shown as gray80 cartoons. The inhibitors and key residues in surrounding are labeled and depicted as cyan and yellow sticks respectively. Hydrogen bonds are shown as red dashed lines.

2.5. Leukemia cells activity

The anti leukemia cells proliferation activities of the inhibitors **3e**, **8h** and **9e** were further evaluated in the MLL-AF4-expressing acute leukemia cell line MV4-11 and the non MLL-rearranged Kasumi-1 cells *in vitro* via cell Titer-Glo luminescent assays (Promega). The parent compound **3a** (IC_{50}=37.1 μM) was used as the positive control. The inhibitors led to a dose-dependent reduction in viable cells with the IC_{50} values of 30.54 ± 1.32, 1.62 ± 0.17, and 1.29 ± 0.32 μM against MV4-11 (Table 1 and Fig S5) and 45.75 ± 3.14, 3.80 ± 0.60, and 4.25 ± 0.04 μM against Kasumi-1 (Table 1 and Fig S6), respectively. It could be seen that the bisamino quinoline inhibitor **3e** was same with **3a** who shown excellent anti-DOT1L activity but exhibited weak activities against the leukemia cells proliferation, perhaps due to their poor cell membrane permeability. The inhibitors **8h** and **9e** possessing the amino side chain inhibited the proliferation of the cancer-cells significantly, indicating that improving the fat solubility of the quinoline inhibitor would be beneficial to their anti leukemia cells activities.

2.6. Cellular H3K79 methylation inhibition analysis

Since compounds **8h** and **9e** exhibited the enhanced anti proliferative activities and poor selectivities against the leukemia cells MV4-11and Kasumi-1, however, compared to **3a** and **3e**, the inconsistency between the cellular and enzymatic assays was observed. To further validate DOT1L as the target, western blot analysis combined with qRT-PCR study[15] were performed to evaluate the direct cellular inhibition activities of these compounds. EPZ004777 was used as the positive control and an equal amount of DMSO was used as the negative control. As shown in Fig 4, **3e** could down-regulate the levels of H3K79 methylation and the gene expression of Hoxa9 and Meis1. The effect was dose-dependent. Similar to **3e**, compound **9e** exhibited moderate effects on Hoxa9 and Meis1 expression down-regulation (Fig 4A). In condition of western blot, **9e** was more effective than **3e**. The dose-dependent phenomenon was observed as well (Fig 4B). In contrast to **3e** and **9e**, **8h** revealed weak down-regulation effect against Hoxa9 and weak inhibition effect on H3K79 methylation at the same condition. Combined the results of other studies together, it could draw a preliminary conclusion that such compounds could target DOT1L in cell to inhibit H3K79 methylation. However, the weak activity of **8h** in cellular H3K79 methylation inhibition studies suggested that DOT1L was one of the potential targets but perhaps not the unique target or the most pivotal one for these compounds, because compounds **8h** and **9e** have the signature chemical features of other inhibitors, such as kinase inhibitors. Other possible targets remain to be uncovered in subsequent exploration.

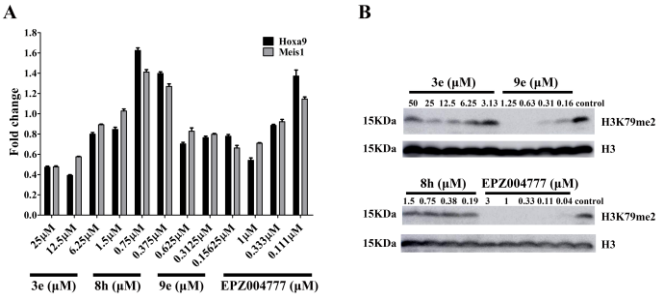

Fig.4 Cellular H3K79 methylation inhibition analysis. EPZ004777 was used as the positive control and an equal amount of DMSO was used as the negative control (A) Hoxa9 and Meis1 expression levels changed after treated with the compounds in indicated concentrations. (B) H3K79 methylation levels were influenced in MV4-11 cells after treated with the compounds in series concentrations.

3. Conclusions

A series of novel pyrimidylaminoquinoline derivatives **8(a-i)** and **9(a-i)** containing amino side chain and the bisamino quinoline analogs **3(b-e)** were designed, synthesized, and evaluated for their DOT1L inhibitory activities. Most of the compounds have strong anti DOT1L activities. Among them, compounds **3e**, **8h** and **9e** are the most potential one from each category, respectively. Compounds **8h** and **9e** exhibited better inhibitory activities against leukemia cells than those of the known inhibitor **3a** and its analog **3e**, suggesting a beneficial role for the newly introduced amino side chain. Additionally,

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compound **9e** could effectively down-regulate both the level of H3k79 methylation and the gene expression of Hoxa9 and Meis1, which demonstrated that DOT1L should be one of the potential targets for these ligands as anti-tumor drug candidates.

Acknowledgments

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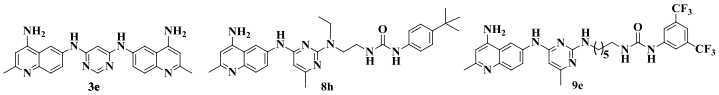
Graphical abstract



3e
IC_{50} (DOT1L) = 1.06 uM
IC_{50} (MV4-11)= 30.54 uM

8h
IC_{50} (DOT1L) = 5.72 uM
IC_{50} (MV4-11)= 1.62 uM

9c
IC_{50} (DOT1L) = 3.55 uM
IC_{50} (MV4-11)= 1.29 uM

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Highlights

> Novel pyrimidylaminoquinoline derivatives containing amino side chain were synthesized.

> Most of the compounds showed strong Dot1L inhibitory activities and binding interactions.

> Compounds **3e**, **8h** and **9e** binds at the SAM binding pocket of DOT1L by molecular docking.

> Compounds **8h** and **9e** showed significant activities against leukemia cells.

> The amino side chain was of benefit to their anti leukemia cells activities.

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