Synthesis of Cyanoenone-Modified Diterpenoid Analogs as Novel Bmi-1-Mediated Antitumor Agents

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Supporting Information

ABSTRACT: Bmi-1 is overexpressed in colorectal cancer (CRC) and served as a novel therapeutic target for the treatment of CRC. A series of novel cyanoenone-modified diterpenoid analogs was synthesized and investigated for their antiproliferative activity against CRC cells. The results showed that most of these compounds exhibited potent antiproliferative and Bmi-1 inhibitory activity. Among them, the most active compound **33** (**SH498**) showed more potent antiproliferative activity than the positive control compound PTC-209. These synthetic diterpenoid analogs were less toxic for normal human fibroblasts (HAF) than for CRC cells. Especially **33**, its selectivity index (SI) between HAF and tumor cells was 7.3–13.1, which was much better than PTC-209. The polycomb repressive complex 1 (PRC1) complex, transwell migration, colony formation, cancer stem cell proliferation, and apoptosis assays of **33** were performed on CRC cell lines. The *in vivo* antitumor effect of **33** was also observed in HCT116 tumor-bearing mice.

KEYWORDS: Diterpenoid, cyanoenone, antitumor, colorectal cancer, Bmi-1

Colorectal cancer (CRC) is the development of cancer from the colon or rectum. It is the third most common cancer and the fourth most common cause of cancer death in the world.¹ There are more than 1 million people that get colorectal cancer around the world every year.² Although the survival of patients with advanced CRC has increased due to improvement in treatment and early diagnosis, considerable treatments still fail to work in many patients because of the metastasis and recurrence. Metastasis and recurrence are the key obstacles to the effective treatment of CRC and remain major clinical challenges. Bmi-1 is a member of the polycomb-group gene family and has been reported as an oncogene in various tumors, which promotes tumor invasion and metastasis.³ Numerous studies demonstrated that Bmi-1 was overexpressed in a variety of cancers,⁴ especially in CRC.⁵ Li et al. reported that the survival rates of CRC patients with low Bim-1 expression were higher than those of patients with high Bmi-1 expression;⁶ thus, Bim-1 is considered a potential and novel therapeutic target for the treatment of CRC.⁷

To the best of our knowledge, there are only a few small molecule inhibitors of Bim-1 that have been reported. PTC-209 (belonging to PTC Therapeutics, Figure 1), which was identified by high-throughput screening of compounds using gene expression modulation by small molecules (GEMS) technology, has been reported as the first preclinical Bmi-1

Figure 1. Chemical structures of Bmi-1 inhibitors.

inhibitor. It was utilized in various cancers including colorectal cancer and other kinds of cancers.^{8–14} In mouse models with patient-derived CRC, the PTC-209 strongly suppressed tumor growth and decreased cancer-initiating cells.⁸ PTC596 (structure not disclosed) also belongs to PTC Therapeutics. It is another potent Bmi-1 inhibitor in multiple tumor cell lines, which was identified in a high-throughput small molecule library screen. PTC596 has entered a Phase 1 clinical trial in patients with advanced solid tumors (NCT02404480).¹⁵ Moreover, rapamycin (Figure 1) is a macrolide produced by the bacteria *Streptomyces hygroscopicus*.¹⁶ A recent study showed that Bmi-1

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was downregulated in protein and mRNA levels by rapamycin treatment in retinoblastoma Y79 cells.¹⁷ The C-28 methyl ester of 2-cyano-3,12-dioxolen-1,9-dien-28-oic acid (CDDO-Me, Figure 1) is a novel synthetic oleanane triterpenoid and has undergone clinical trials for the treatment of solid tumors and lymphomas.¹⁸ CDDO-Me inhibits the proliferation of diverse types of tumor cell lines, including breast, glioblastoma, hepatocellular, leukemia, lung, ovarian, osteosarcoma, pancreatic, prostate, and colorectal cancer cells.^{19–21} Although the mechanisms of the anticancer effects of CDDO-Me are not fully understood, it activates caspase-dependent and -independent apoptosis,²² inhibits MAPK (Erk1/2) and NF- κ B signaling, and modulates TGF- β /Smad and PPAR γ signaling, which contribute to the antitumor activity.^{23,24} Recently, Zhou et al. reported that CDDO-Me also significantly decreased the expression of Bmi-1 in human esophageal squamous cell carcinoma (ESCC) Ec109 and KYSE70 cells.²⁵ Taneja et al. disclosed that the key pharmacophore of CDDO-Me is the cyanoenone group in A-ring.²⁶ Our previous research discovered that a series of novel synthetic tricyclic diterpene analogs possessed potent antiproliferative activity in various tumor cell lines.²⁷ Thus, we expect to produce new Bmi-1 inhibitors by hybridizing the cyanoenone group with our tricyclic diterpene analogs.

Herein, we report the discovery of novel Bmi-1 inhibitors by fusing a cyanoenone group into our previous synthetic antitumor tricyclic diterpene analogs. Their inhibitory activity of Bmi-1 expression was evaluated using the Western blotting assay. Their activity against the growth of colorectal cancer cells and normal human fibroblasts (HAF) was evaluated using the SRB assay. The polycomb repressive complex 1 (PRC1) complex, transwell migration, colony formation, cancer stem cell proliferation, and apoptosis assays were performed on HCT116 cells. The *in vivo* antitumor effect of the most potent compound was also evaluated.

Results and Discussion. Synthesis of Inhibitors. A series of cyanoenone-fused tricyclic diterpene analogs and their intermediates were synthesized according to the pathways described in the Supporting Information (Schemes S1–S3).

The first-round synthetic compound **11**²⁷ and its derivatives **12**, **13**, and **15** were prepared as shown in Scheme S1. Compounds **12**, **13**, and **15** were obtained by modification of the carboxyl group of the mother compound **11** with aromatic amine and alkylamines. The second-round synthetic compounds **16–20** and **22–28** were obtained by modification of the carboxyl group of the mother compound **11** with aromatic amines and phenol and is outlined in Scheme S2. The third-round synthetic compounds **29–34** were obtained by further modifications based on **20** and outlined in Scheme S3. According to the Stork–Eschenmoser hypothesis,^{28,29} all our synthesized cyanoenone-fused diterpenoid analogs belong to racemates.

Biological Results and Discussion. The antiproliferative activity of the first-round synthetic compounds **11–13** and **15** was first screened using the SRB assay in HCT116 colorectal cancer cells (Table 1). The Bmi-1 inhibitory activity was also evaluated using Western blot analysis in HCT116 cells (Figure 2A). Compound **11** and its glycine-modified derivative **15** showed almost no inhibition of cancer cell growth. The methylamine-modified derivative (**12**) and *p*-aminoacetophenone-modified derivative (**13**) possessed potent antiproliferative activity, and **13** ($IC_{50} = 2.10 \mu M$) was 4-fold more potent than **12** ($IC_{50} = 8.54 \mu M$). Western blot analysis showed that only **13** (modification by aromatic amine) had the Bmi-1

Table 1. IC_{50} Values of **11–13** and **15** against the Growth of Colorectal Cancer HCT116 Cells

compd	R	IC_{50} (μM) ^a
11		> 50
12		8.54 ± 0.04
13		2.10 ± 0.03
15		> 50

From SRB assay after 72 h of treatment. ^a IC_{50} data are an average of at least three independent experiments.

Figure 2. Western blot analysis of the protein levels of Bmi-1 in HCT116 cells. After treatment with compounds **11–15** (A) or **13–34** (B) for 8 h, Bmi-1 was detected by Western blot analysis.

inhibitory activity. The results disclosed that introducing aromatic amines in the mother compound **11** may keep both the antiproliferative and Bmi-1 inhibitory activity in tumor cells.

The second-round synthetic compounds **16–20** and **22–28** were evaluated against the growth of colorectal cancer HCT116 cells (Table 2). CDDO-Me and PTC-209 were used as positive controls. The results showed that the antiproliferative activity of all the second-round compounds was more potent than the first-round compounds. In second-round compounds, the phenolic ester substituent (**28**) had weaker antiproliferative activity than the aryl amides (**16–20** and **22–27**). The R substituents of aromatic amines in Table 2 did not show obvious structure–activity relationships. Compound **20**, prepared by introducing the 4-(trifluoromethoxy) aniline substituent, possessed the most potent activity ($IC_{50} = 0.30 \mu M$) in these synthetic compounds of this second-round. Its antiproliferative activity was close to CDDO-Me ($IC_{50} = 0.25 \mu M$) and 2-fold more potent than PTC-209 ($IC_{50} = 0.65 \mu M$), and it was selected for further modification.

The third-round synthetic compounds **29–34** were evaluated against the growth of colorectal cancer HCT116, HT29, and HCT8 cells (Table 3). CDDO-Me and PTC-209 were used as positive controls. The results showed that phenolic hydroxyl group (**29**), which was produced by hydrolyzation of **20**, made the antiproliferative activity decrease dramatically. Similarly, as the 2-cyano group was removed (**34**), the antiproliferative activity was also reduced dramatically. Compared with the substituent 4-(trifluoromethoxy) aniline (**20**), the 4-(trifluoromethoxy) benzylamine (**30**) and 4-(trifluoromethoxy) phenyl hydrazine (**31**) caused the antiproliferative activity to obviously decrease. The activity was slightly attenuated as the substituent 4-(trifluoromethoxy) aniline (**20**) was replaced by 3-(trifluoromethoxy) aniline (**32**). The most potent compound **33** was obtained by condensation of 2-(trifluoromethoxy) aniline with **11**, which possessed more potent antiproliferative activity than

Table 2. IC₅₀ Values of 16–20 and 22–28 against the Growth of Colorectal Cancer HCT116 Cells

compd	R	IC ₅₀ (μM) ^a	Compound	R	IC ₅₀ (μM)
16		0.85 ± 0.21	24		0.82 ± 0.01
17		0.69 ± 0.01	25		0.67 ± 0.10
18		0.57 ± 0.01	26		0.53 ± 0.09
19		0.32 ± 0.12	27		0.76 ± 0.03
20		0.30 ± 0.08	28 ^b		1.31 ± 0.11
22		0.75 ± 0.16	CDDO-Me		0.25 ± 0.01
23		0.56 ± 0.03	PTC-209		0.65 ± 0.03

From SRB assay after 72 h of treatment. ^aIC₅₀ data are an average of at least three independent experiments. ^bX is “NH” in compounds 16–20 and 22–27 and “O” in compound 28.

Table 3. IC₅₀ Values of 20 and 30–34 against the Growth of Colorectal Cancer Cell Lines

compd	R ^a	IC ₅₀ (μM) ^b				SI ^c
		HCT116	HT29	HCT8	HAF	
20		0.30 ± 0.08	0.54 ± 0.01	0.60 ± 0.01	3.22 ± 0.20	5.4–10.7
29		2.00 ± 0.01	2.38 ± 0.08	3.06 ± 0.05	13.77 ± 0.20	4.5–6.9
30		1.04 ± 0.04	1.37 ± 0.03	1.60 ± 0.06	5.19 ± 0.21	3.2–5.0
31		0.90 ± 0.02	1.62 ± 0.05	1.31 ± 0.04	4.55 ± 0.33	2.8–5.0
32		0.43 ± 0.01	0.56 ± 0.02	0.58 ± 0.03	3.57 ± 0.34	6.2–8.3
33 (SH498)		0.25 ± 0.02	0.45 ± 0.01	0.36 ± 0.02	3.28 ± 0.35	7.3–13.1
34		4.56 ± 0.04	4.09 ± 0.03	5.67 ± 0.19	17.13 ± 1.60	3.0–3.8
CDDO-Me		0.25 ± 0.01	0.28 ± 0.01	0.29 ± 0.01	2.10 ± 0.06	7.2–8.4
PTC-209		0.65 ± 0.03	0.61 ± 0.01	0.59 ± 0.01	1.03 ± 0.01	1.6–1.7

From SRB assay after 72 h of treatment. ^aX is methyl group except compound 29 (X = H), Y is CN group except 34 (Y = H). ^bIC₅₀ data are an average of at least three independent experiments. ^cThe selectivity indexes (SI) are calculated by IC₅₀ values in HAF divided by IC₅₀ values in cancer cell lines.

PTC-209. Herein, compound 33 was named SH498. These results illustrated that the C-2 cyano group and C-12 methoxy

group were key substituents for maintaining the potent antiproliferative activity. Compounds with trifluoromethoxy

group in the ortho-position (**33**) of the substituted aromatic ring exhibited more potent activity than its meta- (**32**) and para- (**20**) positions. The Western blot analysis (Figure 2B) showed that Bmi-1 inhibitory activity of these compounds was similar as their antiproliferative activity, most of these compounds, especially **33**, possessed as potent Bmi-1 inhibition as CDDO-Me and PTC-209, except compounds **29** and **34**. The C-2 cyano group and C-12 methoxy group are important to both the antiproliferative and Bmi-1 inhibitory activity in tumor cells.

One of the major hindrances for druggability of candidate compounds is their toxicity to normal cells. Thus, it is important to test cytotoxicity on normal cells in antitumor drug discovery. CDDO-Me, PTC-209, **20**, and the third-round synthetic compounds (**29**–**34**) were chosen for antiproliferative activity evaluating on a normal human fibroblast (HAF) cell line using the SRB assay. The selectivity indexes (SI) were calculated by IC_{50} values in HAF divided by IC_{50} values in colorectal cancer cell lines. The results (Table 3) indicated that these compounds exhibited less toxicity on human fibroblasts in comparison with the tumor cells. The most active compound **33** (SI = 7.3–13.1) showed the highest selectivity toward cancer cells, which was much better than PTC-209 (SI = 1.6–1.7) and slightly higher than CDDO-Me (SI = 7.2–8.4).

Ring1b and Bmi-1 are core subunits of the PRC1 complex, which plays important roles in the regulation of Hox gene expression, X-chromosome inactivation, tumorigenesis, and stem cell self-renewal. Ring1b is an E3 ligase that participates in the ubiquitination of lysine 119 of histone H2A, and the binding of Bmi-1 stimulates the E3 ligase activity.³⁰ We evaluated **33** for reducing PRC1 complex activity by Western blot assay in human colon cancer stem-like cell line HCT116.³¹ As shown in Figure 3, treatment of HCT116 cells with **33** and

Figure 3. Western blot analysis of Bmi-1, H2AK119Ub (ub-H2A), H2A, and Ring1b levels in HCT116 cells. The expression of Bmi-1, ub-H2A, H2A, and Ring1b was detected by Western blot analysis in HCT116 after incubated with indicated concentrations of **33** or PTC-209 for 8 h.

positive compound PTC-209 for 8 h led to a significant reduction of Bmi-1 protein levels in a concentration-dependent manner, which was associated with a decrease of H2A-K119Ub (ub-H2A) and with no effect on total H2A and Ring1b levels, indicating that **33** and PTC-209 specifically reduced PRC1 complex activity by down-regulating Bmi-1 but not Ring1b. We also tested the inhibition ability of PTC-209 and **33** on Bmi-1 protein expression, and the results showed that both compounds displayed similar activity, IC_{50} values of 0.70 and 0.83 μ M, respectively.

Bmi-1 promotes the invasion and migration of colon cancer stem cells.⁷ To determine the antimigration effect of compound **33**, transwell migration assay was performed. As shown in Figure 4A, **33** and PTC-209 decreased migration of HCT116 cells in a concentration-dependent manner. Compound **33** inhibited HCT116 cell migration about 30% at concentration of 0.5 μ M

Figure 4. Compound **33** inhibited HCT116 colon cancer cells migration in transwell chamber (A) and inhibited the colony formation of HCT116 cell line (B). Statistical results are on the right. ** $P < 0.01$; *** $P < 0.001$ versus control (0 μ M).

and almost inhibited the migration about 70% at concentration of 1 μ M compared to the control group (0 μ M), which possessed more potent antimetastatic activity than PTC-209.

Colony formation of tumor cells is closer to its physiology and growth *in vivo*.³² To further determine the antiproliferation effect of compound **33**, colony formation assay was carried out in HCT116 cells. The results (Figure 4B) showed that **33** inhibited colony formation of HCT116 cells significantly in a concentration-dependent manner, including the number and size of colonies. The ability to form colonies was almost completely lost in the presence of **33** at the concentration of 0.4 μ M. In addition, the reduction of colony formation capability of **33** was more potent than the positive compound PTC-209.

Stem-like cancer cells are distinct cellular subpopulations in colon cancer that are essential for tumor maintenance. Bmi-1 plays a critical role in the maintenance of colon cancer stem cells (CSCs). CD133 and CD44 are considered markers of the surface membrane of cells and have been used to identify CSCs.⁷ HCT116 cells coexpressed the putative stem-like cancer cells markers CD133 and CD44.³³ In order to determine whether compound **33** inhibited stem-like cancer cell HCT116 growth, colon cancer stem cells (CD133+, CD44+) were analyzed by flow cytometry. The results discovered that **33** potently suppressed the proliferation of stem-like colon cancer cells in a concentration-dependent manner (Figure S1). The rate of CSCs was decreased obviously from 85.1% to 56.1% after treatment with **33**, which is superior to PTC-209 (from 86.6% to 79.7%).

Compound **33** and PTC-209 were investigated for their potential to induce apoptosis for 48 h using flow cytometry assay (Figure S2). In comparison with untreated controls, **33** (1 μ M) treatment of HCT116 cells generated apoptosis in 20.29% of cells (11.3% early apoptosis and 8.96% late apoptosis), which was more potent than 1 μ M PTC-209 (14.74% apoptosis, containing 9.98% early apoptosis and 4.76% late apoptosis). This result indicated that **33** inhibited cell proliferation through inducing apoptosis in the colon cancer cell line with a concentration-dependent manner.

The subcutaneous tumor model is widely used to imitate the clinical situation of cancer progression in drug research. To

determine the antitumor effect of compound **33** *in vivo*, HCT116 subcutaneous xenograft mouse model was evaluated. Mice were randomly divided into four groups ($n = 6$ per group). Among them, one group was treated with DMSO as a vehicle control, two groups were treated with **33** at 5 and 10 mg/kg/day, respectively, and another group was treated with 10 mg/kg/day PTC-209 as positive group. Nude mice bearing HCT116 tumor cells were intraperitoneally injected compounds and DMSO daily for 18 days, then sacrificed. To our delight, the tumor weight and volume of mice treated with a low dose of **33** (5 mg/kg/day) were much more potently inhibited than mice treated with PTC-209 (10 mg/kg/day) (Figure 5, Figure S3).

Figure 5. Compound **33** prevents tumor cells growth in HCT116 subcutaneous xenograft mouse model. The weight of removed tumors after mice were intraperitoneally injected compounds and DMSO daily for 18 days. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ versus control (0 μ M).

Furthermore, there was no obvious difference in body weight between compound-treated groups and control group (Figure S3). To further evaluate the toxicity of **33** *in vivo*, major organs including hearts, livers, spleens, lungs, and kidneys were analyzed. Histological analysis revealed that **33** had no obvious damage to these major organs of mice (Figure S4). Ki67 (a proliferation marker) and Bmi-1 were also explored by immunohistochemistry in tumors. In comparison with controls, the expression of Ki67 and Bmi-1 were both decreased dramatically in mice groups treated with **33**. Moreover, **33** displayed slightly better inhibition of Ki67 and Bmi-1 than PTC-209 (Figure 6).

Figure 6. IHC analysis of tumors after HCT116 subcutaneous xenograft mice were treated with compound **33**. Arrows showed Bmi-1 and ki67 in the tumor.

Conclusion. A series of novel Bmi-1 inhibitors was disclosed by fusing a cyanoenone group into our previous synthetic antitumor diterpenoid analogs and investigated for their antiproliferative activity of CRC cells. Most of these analogs exhibited potent antitumor and Bmi-1 inhibitory activity on CRC cell lines. The C-2 cyano and C-12 methoxy groups are key substituents to both the antiproliferative and Bmi-1 inhibitory activities. Among them, compounds **33** (**SH498**) showed the highest antiproliferative activity that was more potent than positive control compound PTC-209. The selectivity assay

showed that **33** possessed much more selectivity toward cancer cells than human fibroblasts, which was much better than PTC-209. Mechanism research disclosed that **33** specifically reduced PRC1 complex activity by down-regulating Bmi-1 and ub-H2A. Moreover, the transwell migration, colony formation, and cancer stem cell proliferative ability of CRC cells were significantly inhibited by **33** in a concentration-dependent manner, which was much more effective than PTC-209. Flow cytometry assay indicated that tumor cells death triggered by **33** resulted from apoptotic processes. The *in vivo* antitumor effect in HCT116 tumor-bearing mice also discovered that **33** could inhibit tumor growth effectively with no obvious toxicity. Altogether, **33**, which displayed Bmi-1-mediated significant antitumor effect *in vitro* and *in vivo*, could be used as a promising lead for further development.

■ ASSOCIATED CONTENT

Supporting Information

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Figures, experimental procedures, and characterization data (PDF)

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS

CDDO-Me, 2-cyano-3,12-dioxoolen-1,9-dien-28-oic acid; CRC, colorectal cancer; HAF, human fibroblasts; PRC1, polycomb repressive complex 1; CSCs, cancer stem cells; SI, selectivity indexes

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