Gossypol with Hydrophobic Linear Esters Exhibits Enhanced Antitumor Activity as an Inhibitor of Antiapoptotic Proteins

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Supporting Information

ABSTRACT: A series of gossypol Schiff bases that were derived from unnatural linear amino acid methyl esters were identified and found to be much more potent than gossypol and ABT-199 in terms of anticancer activity. This is the first example of gossypol Schiff bases with increased activity. The investigation of the Schiff base side chain of gossypol revealed that the unique anticancer effect was achieved by the introduction of hydrophobic ester groups. The optimized products showed low micromolar pan antitumor activities against NCI-60 tumor cell lines, which is promising for further drug development. Studies on the preliminary mechanism of action for their cellular activities was also carried out with antiapoptotic protein (Bcl-2 and Mcl-1) inhibition FP assays. The molecular modeling analysis demonstrated a possible binding mode for these compounds with Bcl-2, which could explain the binding affinity of the novel gossypol Schiff bases with these proteins.

KEYWORDS: Gossypol Schiff bases, linear amino acid esters, antiapoptotic proteins

Natural polyphenols have received considerable attention because of their biological activity.¹ Gossypol, a polyphenol that is isolated from cotton seeds, has long been investigated for antitumor activity in addition to the reported antiviral (such as anti-HIV² and anti-H₅N₁³), antifungal,⁴ and antimalarial⁵ activity as well as the inhibition of *trypanosome T. brucei* activity.⁶ (–)-Gossypol is currently in Phase II clinical trials and displays single-agent antitumor activity in patients with advanced malignancies.⁷ The removal or mask of aldehyde groups has been shown to significantly reduce the toxicity of gossypol in humans.⁸ As such, a variety of gossypol derivatives has been developed, including gossypol lactones,⁹ gossypol nitriles,⁵ gossypolone,¹⁰ modification of the main structure of naphthalene,^{7,11} etc. Among these, gossypol Schiff bases displayed widespread biological activities. The purpose of the introduction of the imine through the aldehyde was to reduce the toxicity of gossypol without a loss in the activity since the hydroxyl group appears to be essential for the activity in many cases.¹² However, from known results, the reported activities of aldehyde derived compounds, including gossypol Schiff bases, decreased dramatically compared with gossypol itself in terms of antitumor activity (Figure 1A).^{12–15} The antitumor activities of gossypol Schiff bases were improved slightly only when the framework of gossypol was changed into gossypolone (Figure 1B).¹⁵ The effects of the introduced substituents of gossypol Schiff bases on the antitumor activity were still ambiguous. As a matter of fact, an investigation of the mechanism of the gossypol Schiff bases was not pursued in these previous studies. On the other hand, studies have shown that gossypol may inhibit tumor cells by interacting with Bcl-2 family proteins, which are the central regulators of

Figure 1. Comparison of our newly synthesized Schiff bases with compounds reported to have anticancer activity.

apoptosis (programmed cell-death).¹⁶ The modulation of the antiapoptotic Bcl-2 family (Bcl-2 and Mcl-1) is necessary for protein–protein interactions (PPIs) during apoptosis. Thus, an ideal inhibitor of antiapoptotic proteins should bind to both Bcl-2 and Mcl-1.¹⁷

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Therefore, to address the role of substituents on gossypol Schiff bases, to explore the SAR, and ultimately to discover agents that increase the broad biological activities with the reduced toxicity of gossypol, we systematically investigated many types of gossypol Schiff bases that targeted both Bcl-2 and Mcl-1 (including a small number of previous synthetic compounds that lacked antitumor activity information, see details in [Supporting Information](#)).¹⁸ The results demonstrated that gossypol with hydrophobic linear esters increased antitumor activity and reduced cytotoxicity. This finding is different from the conclusion of increased activity that is induced by hydrophilic effects in the literature.^{14,15}

The results indicated that compound **16** with a hydrophobic linear ester was the most active, and the compound showed pan antitumor activity with GI₅₀ values of 0.1 to 1.7 μ M in NCI-60 human cancer cell lines ([Figure 1C](#)). The therapeutic index of compound **16** was over 30-fold higher than gossypol and showed 10-fold better antitumor activity and 10-fold lower cytotoxicity than gossypol. The competitive FP assays demonstrated that the active compounds had high binding affinity to Bcl-2 and Mcl-1. The results of the molecular modeling study indicated that hydrophobic groups that were introduced by linear amino acid methyl esters were responsible for the extra affinity for the potent compounds.

Cell Viability of Human and Mouse Cancer Cell Lines.

The SAR information on the reported substrates of gossypol Schiff bases is limited and includes few discussions about the effect of the substituents. It was reported that the natural product cryptosphaerolide, with an aliphatic chain, exhibited HCT-116 activity through the Mcl-1 pathway.¹⁹ Thus, we proposed that the introduction of the hydrophobic moiety into gossypol could be beneficial for the activity of the compound. Therefore, we synthesized a set of different types of amines from gossypol that included aromatic amines, aliphatic amines, amino acid derivatives, etc., described in detail in the [Supporting Information](#). The antiproliferative activity of the target gossypol Schiff bases was initially studied with an MTT cell viability assay for human and mouse cancer cell lines. The results are shown in [Table 1](#).

First, compounds **1–6** with different sizes and lengths of aliphatic hydrophobic groups (such as adamantyl and *tert*-octyl) were attached, but only compounds **1** and **3** with medium size groups (*tert*-butyl and cyclohexyl) showed moderate inhibitory activity; others did not exhibit activity in cellular assays. We also checked the anticancer activity of Schiff bases with aromatic hydrophobic groups (compounds **7–11**), which were more potent than aliphatic groups (compounds **1–6**). Compound **7**, with a benzoic acid methyl ester, exhibited micromolar inhibitory activity in all of the four cellular assays. We then investigated several natural amino acid methyl ester groups. Gossypol Schiff bases that were derived from natural amino acids have been demonstrated to exhibit good antiviral activity against HIV-1 and H₅N₁,^{2,3} but their antitumor activity was not prominent. In this regard, compounds **12**, **13**, and **14** were prepared from valine, methionine, and glycine methyl esters, respectively. These ester compounds exhibited better activities than aliphatic (compounds **1–6**) and aromatic (**8–11**) ones. Notably, compound **14** is the first modified imine compound with similar inhibitory activity compared to gossypol in all the assays.

With compound **14** in hand, we next investigated gossypol Schiff bases that were derived from unnatural amino acids/esters with different chain lengths (**15–26**). As shown in [Table 2](#) and [Figure 2](#), in human cancer cell lines MCF-7, DU-145, and HCT-116, gossypol Schiff bases that were conjugated with unnatural amino acid esters (**14–19**) were more potent than the

Table 1. Systematic Evaluation of Gossypol Schiff Bases Using Cell Viability Assays

Comp.	R =	DU-145 IC ₅₀ ^a (μ M)	MCF-7 IC ₅₀ ^a (μ M)	HCT-116 IC ₅₀ ^a (μ M)	CT-26 IC ₅₀ ^a (μ M)
1		17.16 $\pm$ 4.17	>100	>100	12.76 $\pm$ 1.21
2		>100	>100	>100	5.40 $\pm$ 0.33
3		20.65 $\pm$ 1.24	>100	80.30 $\pm$ 1.24	25.25 $\pm$ 1.43
4		>100	>100	>100	21.7 $\pm$ 0.62
5		>100	>100	>100	>100
6		>100	>100	>100	>100
7		20.93 $\pm$ 0.66	24.7 $\pm$ 1.13	37.3 $\pm$ 1.56	21.40 $\pm$ 0.54
8		21.92 $\pm$ 0.43	>100	83.13 $\pm$ 1.45	>100
9		>100	>100	>100	12.80 $\pm$ 0.63
10		29.15 $\pm$ 5.76	>100	8.30 $\pm$ 0.65	>100
11		15.47 $\pm$ 0.70	>100	42.36 $\pm$ 0.77	>100
12		12.14 $\pm$ 1.75	77.34 $\pm$ 2.27	77.10 $\pm$ 1.05	5.85 $\pm$ 0.17
13		17.68 $\pm$ 1.11	>100	0.52 $\pm$ 0.78	>100
14		6.23 $\pm$ 0.35	8.24 $\pm$ 0.57	7.75 $\pm$ 0.99	29.70 $\pm$ 1.23

^aIC₅₀ values were the means of three independent experiments.

corresponding amino acids **20–25**. For the latter compounds, the chain length had little effect on the cell growth inhibitory activities ([Figure 2](#)). For the amino acid methyl esters **14–19**, compound **16** ($n = 3$) exhibited the most potent inhibitory activity ([Figure 2](#)). After the replacement of COOH or COOMe groups by a larger group, such as CONHCH₂Ph in compound **26**, a decreased activity was observed. Compounds **27** and **28**, analogues of the gossypol derivative megosin with different chain lengths, were generated to increase water solubility, but displayed modest inhibitory activity in the four cancer cell lines.²⁰ Almost all the compounds exhibited good inhibitory activity in the mouse colon cell line CT-26 ([Tables 1](#) and [2](#)). For example, compounds **2** (IC₅₀ = 5.4 μ M) and **9** (IC₅₀ = 12.8 μ M) exhibited good results that were absent in the other three cancer cell lines (IC₅₀ > 100 μ M).

To examine whether these novel gossypol Schiff bases were superior to the parent ($\pm$)-gossypols in terms of inhibitory activity, we also prepared (+)-gossypol and (–)-gossypol compounds (see the [Supporting Information](#) chemistry section). Antiproliferative data revealed that the linear chain methyl esters of gossypol Schiff bases **14–19** were more active than the parent ($\pm$)-gossypols and performed better than the corresponding enantiomers ([Table 2](#)). Notably, the newly synthesized compounds significantly affected the growth of MCF-7 and CT-26 cancer cells, in which ($\pm$)-gossypol and even (–)-gossypol were ineffective. Compound **16**, which was the most potent compound (IC₅₀ = 4.39 μ M) that inhibited the growth of MCF-7 cells, was 12-fold more potent than (–)-gossypol (IC₅₀ = 54.0 μ M). Similarly, compound **25**, with an IC₅₀ value of 2.08 μ M, was

Table 2. Further Evaluation of the Optimized Gossypol Schiff Bases with Different Linker Lengths Using Cell Viability Assays

Comp.	R =	DU-145 IC ₅₀ ^a (μM)	MCF-7 IC ₅₀ ^a (μM)	HCT-116 IC ₅₀ ^a (μM)	CT-26 IC ₅₀ ^a (μM)
15		9.47 ± 0.87	5.64 ± 0.89	5.24 ± 1.21	4.25 ± 0.25
16		2.39 ± 0.21	4.39 ± 0.46	2.75 ± 1.01	8.39 ± 0.98
17		7.36 ± 2.05	8.22 ± 1.20	3.38 ± 2.32	16.59 ± 3.01
18		8.56 ± 2.44	11.46 ± 0.52	0.64 ± 0.35	13.75 ± 1.27
19		11.42 ± 1.27	8.95 ± 0.21	13.12 ± 2.58	5.59 ± 0.49
20		34.80 ± 1.33	14.43 ± 2.33	26.78 ± 0.51	48.91 ± 2.52
21		21.61 ± 3.09	19.37 ± 1.19	21.01 ± 3.42	67.28 ± 4.32
22		18.14 ± 0.14	20.03 ± 0.44	32.33 ± 4.36	28.82 ± 5.64
23		20.68 ± 0.75	15.94 ± 1.67	22.16 ± 1.87	4.46 ± 0.58
24		27.48 ± 1.56	17.65 ± 0.65	23.12 ± 0.71	4.54 ± 0.79
25		19.06 ± 0.56	11.48 ± 0.81	17.31 ± 1.92	2.08 ± 1.07
26		22.90 ± 3.97	16.10 ± 3.17	29.80 ± 3.21	47.93 ± 2.01
27		28.87 ± 1.54	15.9 ± 1.22	48.91 ± 1.19	3.19 ± 0.39
28		36.03 ± 1.11	17.72 ± 0.69	43.10 ± 2.65	35.58 ± 0.46
(±)-G ^c	- ^b	7.93 ± 0.32	60.10 ± 0.18	3.03 ± 1.43	46.78 ± 0.77
(+)-G	-	19.17 ± 3.66	75.00 ± 0.55	5.01 ± 0.27	61.90 ± 1.14
(-)-G	-	7.97 ± 1.13	54.00 ± 1.09	3.59 ± 0.76	23.20 ± 0.59

^aIC₅₀ values were the means of three independent experiments. ^b— means no substitution. ^cG'' means gossypol.

Figure 2. Cell viability assays of the unnatural amino acid methyl esters (14–19), their corresponding acids (20–25), and amide 26 in selected tumor cell lines.

11-fold more potent than (–)-gossypol ($IC_{50} = 23.2 \mu M$) in CT-26 cells.

***In Vitro* NCI-60 Human Cancer Cell Lines Assay.** Most of the gossypol Schiff bases in this study (compounds 1-3, 8, 9, 11, 14, 16-20, and 23-28) were also selected for cytotoxicity screening against 60 human tumor cell lines. As anticipated, the data obtained by this cell-based assay correlated well with our previous MTT observations (Tables 1 and 2). Namely, compounds with aliphatic (compounds 1-3) and aromatic substituents (compounds 8, 9, and 11) generally showed poor or

modest inhibitory activities at concentrations up to $10\text{ }\mu\text{M}$ in both experimental approaches (see [Supplemental Table S1](#)). On the contrary, unnatural amino acids/esters derived gossypol Schiff bases with different chain lengths (compounds **14**, **16–20**) showed significant cytotoxicity against human cancer cells. It should be noted that the newly synthesized gossypol Schiff bases that were derived from unnatural amino acid esters were more potent than all unnatural amino acids, similar to the results that are presented in [Table 2](#). Notably, the GI_{50} value for the most potent gossypol Schiff base, compound **16**, which was derived from a γ -aminobutyric acid methyl ester, was less than $1.0\text{ }\mu\text{M}$ for 50 tumor cell lines out of the 60 cell lines that were tested, which was approximately 10-fold more potent than the parent ($\pm$)-gossypol (average $\text{GI}_{50} = 3.1\text{ }\mu\text{M}$) and the known Bcl-2 inhibitor drug ABT-199 ($\text{GI}_{50} = 2.77\text{ }\mu\text{M}$) ([Table 3](#) and [Supplemental Table S2](#)). In

Table 3. Average Cell Growth Inhibition Data for Compounds 14 and 16–18 (GI_{50} , μM) against a Panel of 60 Human Cancer Cells

cell type (number of cell lines)	average cell growth inhibition (GI ₅₀ , μ M) ^a					
	ABT-199	($\pm$)-gossypol	14	16	17	18
leukemia (6)	2.11	2.47	1.88	0.56	0.81	1.03
nonsmall cell lung cancer (9)	2.7	2.85	1.65	0.48	1.05	1.51
colon cancer (7)	2.85	3.19	1.63	0.46	1.00	1.44
cns cancer (6)	3.35	2.97	1.63	0.60	1.27	1.46
melanoma (9)	1.75	3.22	2.84	0.99	1.42	2.35
ovarian cancer (7)	3.33	3.50	1.74	0.59	1.26	2.65
renal cancer (8)	2.6	3.16	2.19	0.82	1.14	2.08
prostate cancer (2)	3.51	ND ^b	1.92	0.41	1.09	1.41
breast cancer (6)	2.77	ND	2.44	0.74	1.08	1.72

^aGI₅₀: concentration required for 50% cell growth inhibition. ^bND: not determined.

addition, in most cases, methyl esters of glycine and 6-aminocaproic acid derived gossypol Schiff bases **14** and **18** showed the same level of cytotoxicity as ($\pm$)-gossypol. However, compared to the ($\pm$)-gossypol 5-amino valeric acid methyl ester that was derived from gossypol, Schiff base **17** significantly affected the growth of several cancer cells, including a leukemia cell line, the nonsmall cell lung cancer HOP-92 cell line, the CNS cancer SNB-19 and U251 cell lines, the renal cancer RXF 393 cell line (see [Supplemental Table S2](#)), etc. Based on the data obtained from NIH and our MTT assays, we proposed that the introduction of a linear amino acid methyl esters side chain could benefit the antitumor activity of gossypol Schiff bases. Cytotoxicity studies that were performed with HEK (human embryonic kidney) 293T and Vero cells showed that most compounds were less toxic than gossypol (see [Supplemental Table S5](#)). In addition, in a separate study compound **16** was found to be stable enough at pH 1–10 and to microsomes ([Supplemental Tables S3 and S4](#)).

Binding Affinity to Bcl-2 and Mcl-1 Proteins. Gossypol is a potent inhibitor of Bcl-2 and Mcl-1.^{16,21} It binds to Bcl-2 and Mcl-1 with K_i values of 0.32 and 0.18 μM , respectively, as determined by competitive fluorescence polarization-based (FP-based) binding assays.⁷ The NIH data and our MTT assay results revealed that our newly synthesized gossypol Schiff bases may have a similar mechanism as the parent compound.

To confirm this point, we evaluated the binding affinity of the selected active compounds for Bcl-2 using FP assays in which

Table 4. Cross-Activity of Gossypol Schiff Bases with Selected Linker Length against Bcl-2 and Mcl-1 in Fluorescence Polarization Assays

Comp	IC ₅₀ ^a (μM) FPA		K _i (μM)	
	Bcl-2	Mcl-1	Bcl-2	Mcl-1
14	0.103 ± 0.019	1.433 ± 0.33	0.005 ± 0.004	0.276 ± 0.06
15	0.286 ± 0.039	2.959 ± 0.847	0.090 ± 0.018	0.579 ± 0.166
16	0.825 ± 0.106	0.947 ± 0.364	0.341 ± 0.05	0.180 ± 0.071
17	0.483 ± 0.111	0.740 ± 0.154	0.182 ± 0.052	0.139 ± 0.03
18	0.840 ± 0.075	1.021 ± 0.166	0.348 ± 0.035	0.195 ± 0.032
20	0.573 ± 0.091	0.521 ± 0.053	0.224 ± 0.042	0.121 ± 0.01
22	0.596 ± 0.095	0.661 ± 0.071	0.235 ± 0.044	0.094 ± 0.014
23	0.458 ± 0.053	0.478 ± 0.036	0.171 ± 0.024	0.085 ± 0.007
(+)-16	0.452 ± 0.016	1.249 ± 0.32	0.167 ± 0.008	0.240 ± 0.061
(-)-16	0.375 ± 0.031	0.937 ± 0.175	0.131 ± 0.014	0.178 ± 0.033
gossypol	1.191 ± 0.667	0.856 ± 0.332	0.511 ± 0.312	0.162 ± 0.065
ABT-199	<0.001	>4	<0.001	>0.78

^aIC₅₀ values were the means of three independent experiments.

Triton X-100 was added as a detergent to prevent the possible aggregation of hydrophobic compounds (Table 4 and Figure 3A).

Figure 3. Competitive binding curves of 14, 16, 20, 22, and (±)-gossypol to (A) Bcl-2 and (B) Mcl-1, as determined with the fluorescence-polarization-based binding assay.

The β -alanine methyl ester derived gossypol Schiff base 15 displayed a high binding affinity for Bcl-2, with a K_i value of 0.090 μ M, and the glycine methyl analogue 14 showed an increased binding affinity, with a K_i value of 0.005 μ M. The other ester compounds 16–18 and amino acid derivatives 20, 22, and 23 exhibited similar binding affinities with K_i values that ranged from 0.171 to 0.348 μ M, and all were better than (±)-gossypol (K_i = 0.511 μ M) (Figure 3A). As mentioned above in the MTT assay, unnatural amino acid methyl esters that were derived from the gossypol Schiff bases 14–18 displayed potent efficacy in the inhibition of cell growth in the MCF-7 cell line, which expresses moderate levels of Bcl-2 and Mcl-1 (Table 2).^{22,23} This observation correlated with the binding data of compounds 14–18 for Bcl-2, which indicated that Bcl-2 was the potential target of these compounds. For the enantiomers of the optimized compound 16 (Supporting Information), both of the binding affinities of (–)-16 (K_i = 0.131 μ M) and (+)-16 (K_i = 0.167 μ M) were higher than (±)-16 (K_i = 0.341 μ M) for Bcl-2. Although gossypol Schiff bases 20, 22, and 23 that were derived from the amino acids displayed strong binding affinity (K_i = 0.224, 0.235, 0.171 μ M, respectively) to Bcl-2 in the FP assays, they showed a 2.1-fold lower efficacy in the inhibition of the growth of the MCF-7 cell line, with an average IC₅₀ of 16.8 μ M, compared to the corresponding ester analogues 14–19 with an average IC₅₀ value of 7.82 μ M. The ester analogues 14–19 are also 7.7 times more potent than gossypol (IC₅₀ = 60.10 μ M). This discrepancy is likely due to the high hydrophilicity of these unnatural amino acids, so they always exhibit poor cell membrane permeability compared to the corresponding esters. Therefore, the gossypol unnatural amino acids 20–23 displayed good binding affinity to Mcl-1 and

Bcl-2 proteins, and they always showed relatively weaker efficacy in the inhibition of cancer cells. It was also reported the high hydrophilicity of apogossypolone derivatives could result in a low cell permeability and would therefore exhibit moderate antitumor activity, although those compounds displayed good binding affinity to Bcl-2 family proteins.²⁴

Next, we evaluated the binding properties and specificity of selected gossypol Schiff bases against Mcl-1 using FP assays (Table 4); Mcl-1 was expressed at high levels in many cancer cell lines such as the colon cancer HCT-116²⁵ and prostate cancer DU-145²⁶ cell lines. The cell growth inhibition of these compounds in these two cell lines is shown in Tables 1 and 2. All of the tested compounds displayed superior affinity against Mcl-1 in the FP assays, similar to that of gossypol (K_i = 0.162 μ M) (Figure 3B). Consistent with binding data, (±)-gossypol exhibited potent efficacy in the inhibition of cell growth in HCT-116 and DU-145 cell lines according to the results of the MTT assay, with IC₅₀ values of 3.03 and 7.93 μ M, respectively (Table 2). Other unnatural amino acid methyl esters that were derived as gossypol Schiff bases 14–18 also displayed strong inhibitory properties against the Mcl-1 protein. The most potent compound, compound 17, displaced BH3 binding to Mcl-1, with IC₅₀ values of 7.36 and 3.38 μ M to DU-145 and HCT-116, respectively (Table 2). However, the compounds 20, 22, and 23 displayed very high binding affinity to Mcl-1 in the FP assays, with average IC₅₀ values of 0.10 μ M; the compounds showed a relatively weak efficacy in inhibiting the growth of DU-145 and HCT-116 cell lines, with average IC₅₀ values of approximately 24.54 and 27.09 μ M, respectively. The lower IC₅₀ of cellular data of compounds 20–23 with higher Mcl-1 binding affinity may be due to a permeability issue, as observed in Bcl-2.

To investigate the activities and mechanisms of gossypol imines, ABT-199 was selected as a control compound. ABT-199 is known as a highly Bcl-2 selective antagonist. Our data revealed that compound 16 had lower Bcl-2 binding affinity than ABT-199 but had higher Mcl-1 affinity. According to the NCI cell line data, ABT-199 exhibited the highest activity in the leukemia cancer cell line HL-60, which expresses high levels of Bcl-2. In addition, compound 16 exhibited an equal potency to ABT-199 in the HL-60 cell line (Supplemental Table S2). Furthermore, compound 16 was more potent in most human cancer cell lines than ABT-199 (Supplemental Table S2). Mcl-1, but not Bcl-2, is expressed in most human cancer cell lines.²⁶ Thus, it was possible to infer

that the gossypol Schiff bases were superior to ABT-199 via the inhibition of both Bcl-2 and Mcl-1 mechanisms.

With the combination of binding affinities and all the *in vitro* cell viability data, one can conclude that gossypol Schiff bases that were derived from unnatural linear amino acid methyl esters could enhance the antitumor activity of gossypol. Furthermore, the chain length is also important for Bcl-2 family protein binding; the data revealed that the three-carbon linker was the best in the series. Studies have indicated that small molecular inhibitors of the Bcl-2 family with longer, flexible hydrophobic groups have displayed higher potencies than small, short, and rigid hydrophobic groups.²⁷ Therefore, computational docking studies were carried out to understand the binding model of the selected compounds to Bcl-2 family proteins and the SAR of the different substituents.

Molecular Modeling. The enamine form of the gossypol Schiff base analogue was confirmed by X-ray crystal structure,⁴ so we used this form in our molecular model study. One advantage of the gossypol enamine tautomer is that it is stabilized by intramolecular hydrogen bonding. Compound (–)-16 was chosen to be studied by molecular docking since the (–)-gossypol conformation exhibits a better binding affinity than the corresponding (+)-conformation.

As observed in Figure 4A,B, the aliphatic chain could occupy the P2 and P3 pockets of the Bcl-2 protein without the flexible

Figure 4. (A,B) The interaction models of compound (–)-16 with Bcl-2. (A) The binding site and orientation of the compound (represented by compound (–)-16) in the hydrophobic groove P2 and P3 of Bcl-2. (B) The protein is rendered in a surface model, while the compound is rendered in a stick model and a van der Waals sphere (CPK) model. (C,D) Two possible poses were assessed in the molecular docking study, (C) with no ligand–protein hydrogen bonding interaction and (D) with a ligand–protein hydrogen bonding interaction; Bcl-2 is rendered as a cartoon, while the compound and the residues around it are rendered as sticks (enlarged pictures, see Supplemental Figure S11).

loop domain in the model.²⁸ The size and orientation could fit the pocket quite well in the ligand CPK model. We then analyzed the details of the compound (–)-16 and the protein interaction from the molecular modeling point of view.

The major surface of the binding groove is a hydrophobic region that includes A97, F101, M112, N149, and F150 and the polar residue rich region that contains R104, D108, R143 and Y199. The helices, which contain Y199 and D100, were observed to have strong H bonding according to X-ray structure data and formed the wide hydrophobic pocket P3 for the peptide or ligand interaction. R143 was found to interact with D of the BIM peptide through H bonding. However, in the reported small molecule and Bcl-2 complex, hydrogen bond interactions with the receptor

were observed less often, except in one example in which the ABT-199 indole analogue interacted with D100.²⁹ In our cases, two possible binding models are presented in Figure 4C,D; one model included the most observed hydrophobic interaction without the protein–ligand hydrogen bonding interaction, and the other model used the hydrogen bonds between the hydroxyl group of gossypol and the nitrogen of R143 as well as the oxygen of the carbonyl group of gossypol and the nitrogen of R104. We used AmberScore to evaluate the binding energy because AmberScore has been shown to be one of the most precise MD scoring tools for docking studies.³⁰ The first model had a much lower score, –24, than the second model, which had a score of –14 based on the AmberScore. In contrast, the score of gossypol in the form of (–)-16 was –14, as shown in Figure 4D; this score was lower than the form that is presented in Figure 4C. The amino acid ester chains that are shown in Figure 4D did not contribute extra binding energy. The hydrophobic interaction could therefore improve the interaction between the receptor and the ligand, even when the H bonding interaction was sacrificed. These results are also consistent with the following findings: from BIM to different types of peptides (stapled or unnatural), the four hydrophobic residues h1 to h4 (ILIF) were widely applied to design novel peptide inhibitors.³¹ The activity of gossypol imines was improved by the hydrophobic interaction that resulted from the introduction of the aliphatic side chains.

In summary, a series of gossypol Schiff bases with linear unnatural amino acids/methyl esters were demonstrated to be potent inhibitors of antiapoptotic proteins. Compound 16 was found to bind to Bcl-2 and Mcl-1 with IC₅₀ values of 0.341 and 0.180 μ M, respectively. The *in vitro* cellular assay indicated that compound 16 potently inhibited the growth of cancer cells. The cell growth inhibition data from the NIH were consistent with our results, and the GI₅₀ values of compound 16, the most potent compound, ranged from 0.1 to 1.7 μ M, which were better than the values that were obtained with gossypol and ABT-199.

■ ASSOCIATED CONTENT

§ Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsmedchemlett.6b00302.

Experimental procedures and chemical data for final compounds, molecular modeling, biochemical assays, and stability assays. NCI-60 one dose (10 μ M) screening data for compounds 1–3, 8, 9, 11, 14, 16–20, 23–26, and 28. Details of NCI-60 information for compounds ABT-199, 14, 16, 17, and 18 (PDF)

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Notes

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■ ABBREVIATIONS

SARs, structure–activity relationships; PPIs, protein–protein interactions; MTT, 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide; CNS, central nervous system; Bcl-2, B-cell lymphoma 2; Mcl-1, myeloid cell leukemia 1; FP, fluorescence polarization; CPK, Corey–Pauling–Koltun

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