Structure-based Design, Synthesis, And Evaluation of a New Bcl-2/Mcl-1 Inhibitor

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Abstract. Based on our previously reported Bcl-2/Mcl-1 dual inhibitor S1, we designed a new Bcl-2/Mcl-1 inhibitor through a structure-based scaffold-hopping approach, replacing the 6-thiomorpholine of S1 by piperazine group for P2 occupation. Compound S1-11 was synthesized readily by nucleophilic substitution reaction between 1-oxo-1H-phenalene-2,3-dicarbonitrile (OPD) with piperazine, and exhibited potent binding capability to both Bcl-2 (Ki=0.82 ± 0.06 μM) and Mcl-1 (Ki=0.43 ± 0.01 μM). Furthermore, S1-11 exhibited potent lethality on MCF-7 cells.

Keywords: Apoptosis, Bcl-2/Mcl-1 protein, Inhibitor.

1. Introduction

Programmed cell death, also called apoptosis, is an important regulatory mechanism for the removal of damaged, aging, and unwanted cells [1-2]. Bcl-2 family proteins, including proapoptotic members (Bax, Bak), antiapoptotic proteins (Bcl-2, Mcl-1, and Bcl-xl) and BH3-only proteins (Bim, Puma, Bid, Bad, Noxa, etc.), are the key regulators of apoptosis [3-4]. The dynamic interactions between the BH3 domains in proapoptotic and BH3-only proteins and the BH3 groove in antiapoptotic proteins determined the balance between apoptosis and survival. Studies have shown that antiapoptotic Bcl-2 family proteins are always overexpressed to block apoptosis in tumor cells [5-6], which is very crucial for tumorigenesis, maintenance, and drug resistance [7]. Thus, it is a useful strategy to develop drugs that could target the pro-survival members of Bcl-2 family to fight against cancer [8-9].

During the past decade, much more efforts has been made to the discovery of inhibitors targeting Bcl-2 family [10-11]. Recently, ABT199 [12], the most promising Bcl-2 inhibitor, was approved by FDA and it was used to the therapy of chronic lymphocytic leukemia (CLL). However, the cancer killing ability of ABT-199 is limited. It only has effects on tumors which were depended on Bcl-2 to survive, and it will meet with drug-resistance in cancer cells overexpressing Mcl-1 protein [13].

Previously, we have reported a Bcl-2/Mcl-1 dual inhibitor, 4-thiomorpholinyl-2, 3-dicyanophenanone (S1). As determined by ^{15}N, 1H nuclear magnetic resonance (NMR) titration, and structure–activity relationship (SAR) study [14-15], S1 occupies the p2 pocket in the BH3 groove of both Bcl-2 and Mcl-1. Based on S1, we developed 4-thiomorpholinyl-2-cyano-3-amidaminephenanone which was capable of occupying p2 and p3 [16]. Herein, we further designed and synthesized a new Bcl-2/Mcl-1 inhibitor by replacing the 6-thiomorpholine by piperazine group for P2 occupation. S1-11 exhibited the most potent binding affinities to both Mcl-1 and Bcl-2 proteins, with Ki values in the sub-micromolar range. Furthermore, S1-11 exhibited lethality and showed the same order of affinity as (-)-Gossypol and S1 on MCF-7 cells.

2. Experimental

2.1. Material and Methods

All solvents and reagents involving in experiments were purchased from commercial suppliers and directly used without any further purification. The solution of S1-11 was dissolved in DMSO at a concentration of 10 mM as the stock solution. 1H NMR spectra were recorded on a Bruker 500

spectrometer. Mass spectrometric data were achieved with HP1100LC/MSD MS and an LC/Q-TOF-MS instruments.

2.2. Fluorescence Polarization (FP) Assay

For the competitive binding assay for the Mcl-1 and Bcl-2 protein, FAM-Bid peptide (10 nM), Mcl 1 protein (55 nM), and Bcl-2 protein (140 nM) were preincubated in the assay buffer (100 mM potassium phosphate, pH 7.5; 100 μg/mL bovine gamma globulin; 0.02% sodium azide). Next, serial dilutions of compounds were added. After a 30 min incubation, the polarization values were measured using the Spectra Max M5 Detection System in a black 96-well plate. Saturation experiments determined that FAM-Bid binds to the Mcl-1 and Bcl-2 proteins with Kd values of 1.9 and 8 nM, respectively. The Ki value for each inhibitor was calculated using the equation: $Ki = [I]_{50}/([L]_{50}/Kd+[P]_0/Kd+1)$ where $[I]_{50}$ denotes the concentration of the free inhibitor at 50% inhibition, $[L]_{50}$ is the concentration of the free labeled ligand at 50% inhibition, $[P]_0$ is the concentration of the free protein at 0% inhibition, and Kd is the dissociation constant of the protein ligand complex.

2.3. Cell lines

MCF-7 were cultured in DMEM/high glucose supplemented with 10% fetal bovine serum (Gibco Company, USA) and streptomycin was used to culture the cells at 37°C and 5% CO_2 [17].

2.4. Annexin-V Staining Assay

Annexin-V apoptosis detection kit (Roche, Indianapolis, IN) was used to detect apoptosis and the procedure was conducted according to the manufacturer's instructions. For specific experimental details refer to our previous reports [17].

2.5. Synthesis

OPD (1.0 eq.) was dissolved in 25 mL acetonitrile, and then piperazine (2.0 eq.) was added to the reaction system and stirred at room temperature for 3 h. After the full reaction of OPD, the solvent was removed by steam. The crude product was separated by silica gel column with dichloromethane: petroleum ether (v/v, 100:1) as eluent, and the active molecule S1-11 was obtained. Yield: 38 %.^{1}H NMR (500 MHz, DMSO-d6) δ 8.75 (d, J = 7.5 Hz, 1H), 8.56 (d, J = 8.2 Hz, 1H), 8.26 (d, J = 8.3 Hz, 1H), 7.89 (t, J = 7.8 Hz, 1H), 7.16 (d, J = 8.2 Hz, 1H), 3.92 (s, 4H), 3.67 (s, 4H). ESI-MS: m/z $C_{19}H_{14}N_4O$, $[M+H]^+$ calculated 314.12, found 314.1.

3. Results and Discussion

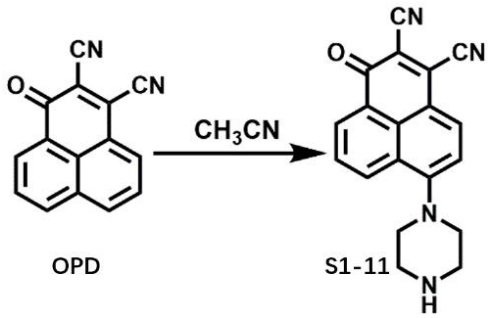

Figure 1. Synthesis of S1-11

Previously, we optimized a series of N-substituted piperazine groups based on a Bcl-2/Mcl-1 dual inhibitor scaffold S1 and the substituting groups occupy the P2 pocket to increase affinity with the target protein. In this study, we introduced a new Bcl-2/Mcl-1 inhibitor, which was achieved by substituting the 6-thiomorpholine with a piperazine group for P2 occupation. Compound S1-11 was synthesized readily by nucleophilic substitution reaction between 1-oxo-1H-phenalene-2, 3-

dicarbonitrile with piperazine (Figure 1). The structure of compound S1-11 was confirmed by 1H NMR and MS.

Table 1. Binding affinities (by FPAs) of compounds to Mcl-1/Bcl-2

Compound		FPAs [Ki ± SD (μM)] [b]	
		Bcl-2	Mcl-1
(-)-Gossypol [a]		0.45 ± 0.10	0.20 ± 0.05
S1		0.31 ± 0.12	0.06 ± 0.18
S1-11		0.82 ± 0.06	0.43 ± 0.01

[a] (-)-Gossypol was obtained from Selleck, China.
[b] Values are the mean ± standard deviation of three independent experiments.

Subsequently, the binding affinity was evaluated through fluorescence polarization assay (FPA), using Bcl-2/Mcl-1 dual inhibitor (-)-Gossypol and S1 as control. As depicted in Table 1, S1-11 demonstrated a potent binding capability to both Bcl-2 (Ki=0.82 ± 0.06 μM) and Mcl-1 (Ki=0.43 ± 0.01 μM). Molecular docking simulation further elucidated that the piperazine group of S1-11 protruded into the P2 pocket like the thiomorpholine group of S1(Figure 2) [18].

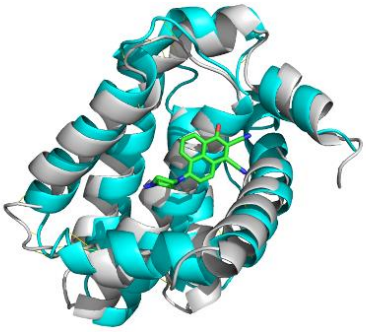

Figure 2. Predicted binding models of S1-11 (green) in complex with Bcl-2/Mcl-1 (PDB ID: 2XA0 for Bcl-2 and 2NLA for Mcl-1)

Next, the cytotoxic activity of S1-11 was assessed on MCF-7 cells, which rely on antiapoptotic Bcl-2 family proteins for survival. Cells were incubated with a gradient concentration of S1-11 for 72 hr, following which annexin-V staining was carried out to ascertain the apoptosis rate (Figure 3). Commercial Bcl-2/Mcl-1 dual inhibitors (-)-Gossypol and S1 was used as a positive reference. S1-11 potently induced apoptosis, showing a potent lethality in MCF-7 cells.

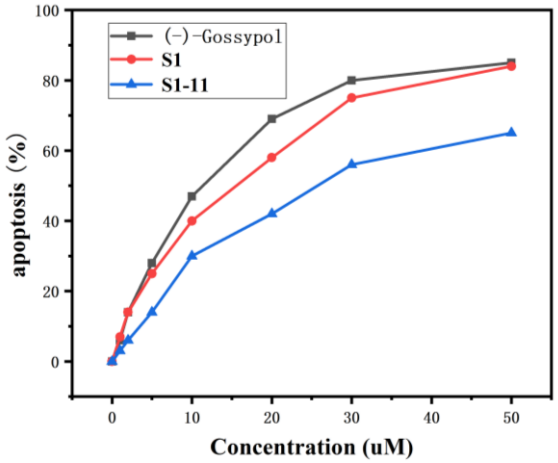

Figure 3. Cell apoptosis assays of MCF-7 cells upon adding S1-11, S1 and (-)-Gossypol as determined by annexin-V staining

4. Conclusion

In summary, a novel Bcl-2/Mcl-1 inhibitor was designed through a structure-based scaffold-hopping approach from the previously identified Bcl-2/Mcl-1 dual inhibitor S1. Compound S1-11 was synthesized facilely by nucleophilic substitution reaction between 1-oxo-1H-phenalene-2,3-dicarbonitrile with piperazine, and exhibited potent binding capability to both Bcl-2 (Ki=0.82 ± 0.06 μM) and Mcl-1 (Ki=0.43 ± 0.01 μM). Moreover, S1-11 displayed potent lethality effect on MCF-7 cells.

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