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Anti-tumor pyrimidinylpiperazines bind to the prosurvival Bcl-2 protein family member Bcl-XL

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ABSTRACT

Overexpression of prosurvival or underexpression of pro-death Bcl-2 family proteins can lead to cancer cell resistance to chemotherapy and radiation treatment. Inhibition of the prosurvival Bcl-2 family proteins has become a strategy for cancer therapy and inhibitors are currently being evaluated in the clinic both as single agents and in combination with established drugs. Here we describe the design, synthesis, and evaluation of pyrimidylpiperazines that were discovered to be inhibitors of the prosurvival Bcl-2 protein family member Bcl-XL. This study identified compound **21** which demonstrated a GI_{50} value of 8.4 μM against A549 lung adenocarcinoma cells and a binding affinity K_i value for Bcl-XL of 127 nM.

Molecules that can selectively kill tumor cells have great potential as drug candidates for the treatment of various cancers. One strategy for the development of potential drug molecules that can selectively kill tumor cells is the modulation of the apoptosis process. Apoptosis, sometimes referred to as programmed cell death, is the process by which cell homeostasis is maintained and by which devastating genetic abnormalities are dealt.

The B-cell lymphoma-2 (Bcl-2) protein family plays an important role in the regulation of apoptosis in mammalian cells.[1] Apoptosis control is characterized by a delicate balance between homo- and hetero-dimerization of pro- and anti-apoptosis members of the protein family. Inhibiting this protein–protein interaction is one viable approach to cancer therapy.[2] Anti-apoptosis (prosurvival) family members Bcl-2, Mcl-1, and Bcl-XL are current targets for anti-cancer drug design.

The Bcl-XL protein is an anti-apoptotic member of the Bcl-2 family and acts by forming a hetero-dimer with, and thus inhibiting the function of pro-apoptosis activator proteins including Bid and Bim. This association is mediated through the binding of a BH3 domain helix of Bid and or Bim to a hydrophobic cleft on the surface of Bcl-XL. The function of pro-apoptosis activator proteins is to form a hetero-dimeric complex with pro-apoptosis Bcl-2 family members Bak or Bax and initiate the release of cytochrome c (Cyt c) from the inner membrane of the mitochondria. Cytochrome c induces the formation of the apoptosome with subsequent activation of caspase-9 followed by caspases-3 and -7 that degrade various cellular targets and cause cell death.[3]

There are drug leads currently in the developmental pipeline that target Bcl-2 anti-apoptotic protein family members as well as drug candidates in current clinical trials. Gossypol,[4] other polyphenols, and antimycins[5] are natural product small molecules known to bind to Bcl-2 and/or Bcl-XL that are currently being evaluated in clinical trials for B-cell malignancies and breast cancer chemoprevention, respectively. Apogossypol[6] and TW-37[7] are designed small molecule inhibitors of Bcl-2 family proteins based upon gossypol. Fragment based design methods have produced the acylsulfonamide class of inhibitors,[8] isosteric replacement based analogs,[9] and thiazolidin derivatives.[10] Obatoclax[11] is in clinical trial for hematological malignancies and ABT-263,[12] an acylsulfonamide, is being evaluated in trials for CLL and advanced small cell lung cancer.

The existence of numerous PDB entries for complexes of Bcl-XL bound to peptide and various ligands provided an excellent opportunity for structure-based design of potential new ligands. Bcl-XL sequesters Bak, Bim, Bid, and other pro-death Bcl-2 protein family members by binding of their BH3 helix in a groove on the surface of Bcl-XL. The binding groove on the surface of Bcl-XL is quite large and may be divided into three regions: (1) A deep narrow binding site (site 1). (2) A broad shallow binding site (site 2). (3) An aromatic ridge that separates site 1 from site 2. Figure 1 depicts the binding site on Bcl-XL empty (left) and occupied by one of our designed ligands **22** (right). Studies have shown that molecules that bind tightly to their protein targets are more often 'needle-like'

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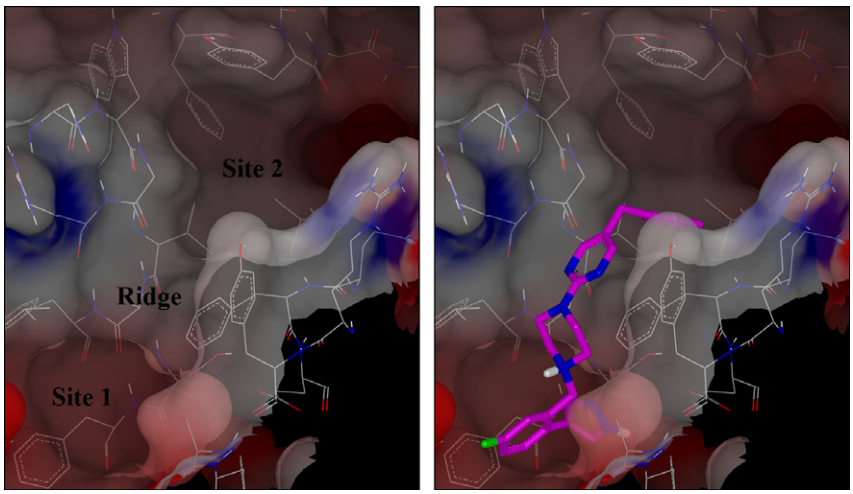


Figure 1. Close-up view of X-ray derived crystal structure of Bcl-XL binding site alone (left) and docked with **22** (magenta, right).

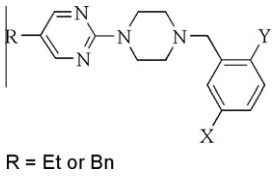
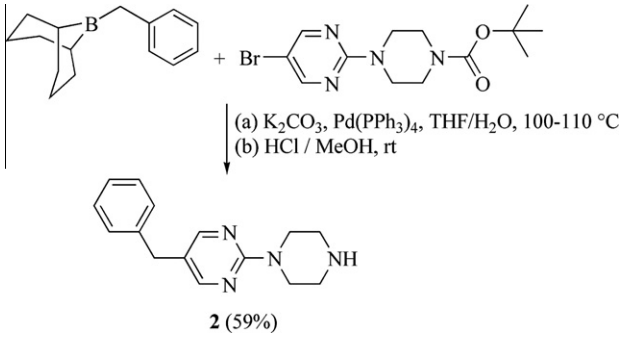


Figure 2. General structural formula of pyrimidinylpiperazines.

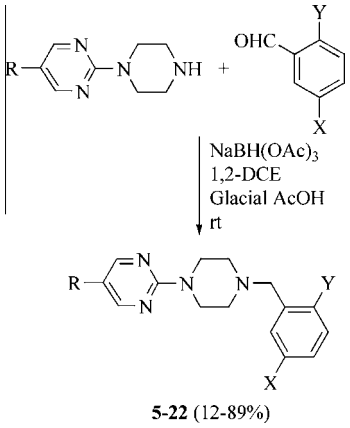
and bind to deep pockets.[13] These observations led us to reason that most of the binding affinity of a drug molecule bound to Bcl-XL will likely come from targeting the lower deep narrow binding site (site 1).

Based on these observations and molecular docking experiments we designed a series of molecules with a pyrimidinylpiperazine core which primarily target site 1 (Fig. 2). The 5-position of the pyrimidine is substituted with either an ethyl or benzyl group. The site 1 binding group was varied with N-benzylic substituents. The piperazine ring system is attractive for its symmetry, nitrogen atom content, and low molecular weight. From a synthetic point of view the binucleophilic character of piperazine makes it easy to derivatize, and to design multiple series of molecules. Schemes 1 and 2 exhibit the synthesis of these basic core molecules **1** and **2**. Commercially available starting materials were converted to **1** and **2** in two steps. A nucleophilic aromatic substitution reaction followed by removal of the Boc group provided **1** (Scheme 1). A Suzuki cross-coupling reaction followed by removal of the Boc group provided **2** (Scheme 2).[14]

Molecules **1** and **2** were derivatized to provide the *N*-benzyl series. Reductive amination with aromatic aldehydes gave molecules **5**–**22** (Scheme 3).[15] Transformation of the nitro derivatives **10**, **11**, **19**, and **20** to the corresponding amino derivatives **23**–**26** was problematic. Attempted hydrogenation with H_2 and Pd/C resulted in significant debenzylation of the piperazine nitrogen. Reduction using the $SnCl_2$ method was messy and difficult to workup.[16] Ultimately reduction with iron(II) sulfide provided clean conversion to

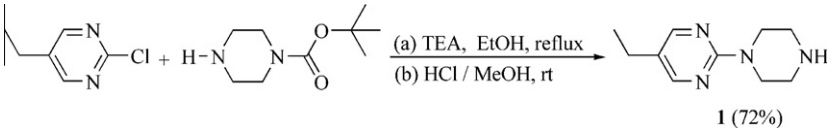


Scheme 2. Chemical synthesis of intermediate **2**.

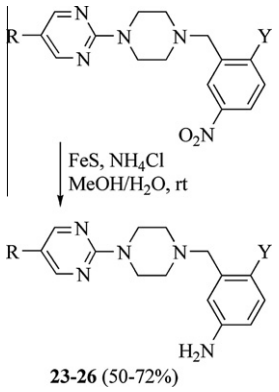


Scheme 3. Chemical synthesis of final compounds **5**–**22**.

the desired products (Scheme 4).[17] Molecules **12**, **13**, **21**, and **22** required the aromatic aldehydes **3** and **4** which were prepared via



Scheme 1. Chemical synthesis of intermediate **1**.



Scheme 4. Chemical synthesis of final compounds **23**–**26**.

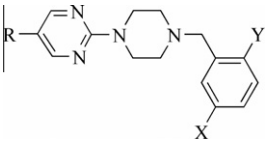
Suzuki cross-coupling reaction according to Scheme 5. Synthetic intermediates and final products were characterized by NMR (^1H and ^{13}C) and mass spectrometry, and the purity of final products was determined by analytical HPLC (see Supplementary data).

The molecules were screened against human non-small cell lung tumor A549. The A549 cell line expresses significant amounts of Bcl-XL protein and is moderately sensitive to Bcl-XL inhibitors.[18] In order to identify active molecules, we initially screened at a single high dose of 100 μM using the sulfarhodamine B (SRB), 48-h growth inhibition assay.[19] The SRB assay identified several molecules as having significant activity at this concentration (data not shown). This initial screen was followed up with determination of these molecules' GI_{50} values. The GI_{50} values against the cell line are shown in Table 1. Additionally, we confirmed that the cell line used for screening expressed Bcl-XL by immunoprecipitation. Representative active molecules were then evaluated in a competitive protein binding assay. Molecules **17**, **21**, and **26** were subjected to a binding competition for Bcl-XL with a fluorescent BAK BH3 peptide.[20] The inhibition constants (K_i) for these molecules are reported in Table 2.

The prepared pyrimidinylpiperazines exhibit inhibitory activity against the A549 lung adenocarcinoma cell line. The GI_{50} values for this cell line make it evident that the benzyl series is more potent than the ethyl series. From this observation it is surmised that the hydrophobic aromatic group is necessary at the 5-position of the pyrimidine ring in the molecule for either binding to the biological target or efficient penetration of the cell. The most active molecules bear a hydrogen bond donor/acceptor group and a lipophilic (halogen or pyrazole ring) group on the common N-benzylic substituent in both series.

The pyrimidinylpiperazines have a relatively small ethyl or benzyl group that accesses but does not fill site 2, yet these molecules still bind to Bcl-XL in the mid-nM range. Figure 1 demonstrates the predicted binding geometry of **22** in the binding site of Bcl-XL. From the predicted docking pose, it is hypothesize that the pyrimidinylpiperazine core of **22** adopts a conformation that complements the contour of the binding site and that the benzyl group at the 5-position of the pyrimidine just accesses binding site 2.

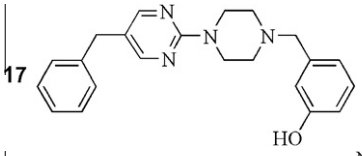
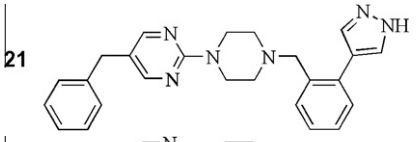
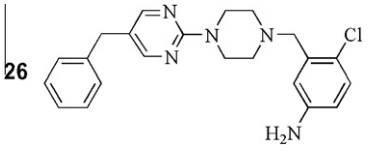
Table 1
Growth inhibition of A549 lung adenocarcinoma cells by *N*-benzyl pyrimidinylpiperazine analogs



Compound	R	X	Y	GI_{50} ± SEM[a] (μM)
5	Ethyl	H	H	>100
6	Ethyl	H	Br	70.9 ± 2.57
7	Ethyl	F	Br	>100
8	Ethyl	OH	H	>100
9	Ethyl	OH	Br	57.6 ± 48.7
10	Ethyl	NO_2	H	88.0 ± 4.25
11	Ethyl	NO_2	Cl	67.2 ± 4.99
12	Ethyl	H	1*H*-Pyrazol-4-yl	51.4 ± 2.01
13	Ethyl	F	1*H*-Pyrazol-4-yl	49.2 ± 0.95
14	Benzyl	H	H	49.6 ± 4.85
15	Benzyl	H	Br	86.5 ± 42.3
16	Benzyl	F	Br	>100
17	Benzyl	OH	H	51.1 ± 0.70
18	Benzyl	OH	Br	23.7 ± 1.78
19	Benzyl	NO_2	H	61.2 ± 15.5
20	Benzyl	NO_2	Cl	>100
21	Benzyl	H	1*H*-Pyrazol-4-yl	8.43 ± 2.02
22	Benzyl	F	1*H*-Pyrazol-4-yl	9.91 ± 1.18
23	Ethyl	NH_2	H	>100
24	Ethyl	NH_2	Cl	62.9 ± 1.61
25	Benzyl	NH_2	H	75.4 ± 4.16
26	Benzyl	NH_2	Cl	15.1 ± 2.06

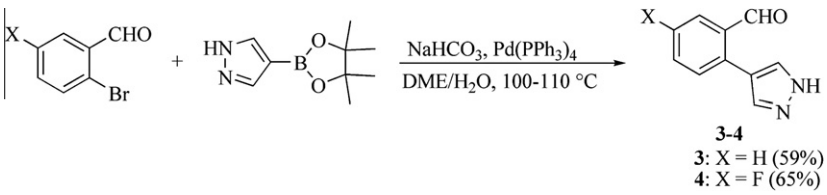
[a] Values are the average of two experiments done in triplicate.

Table 2
Binding affinities of select pyrimidinylpiperazines for Bcl-XL

Compound	K_i ± SEM[a] (μM)
17 	0.52 ± 0.21
21 	0.127 ± 0.04
26 	0.170 ± 0.07

[a] Values are average of three experiments.

The favorable shape complementarity of **22** and Bcl-XL allows the *N*-benzyl substituent to penetrate deep into the site 1 binding



Scheme 5. Chemical synthesis of intermediates **3** and **4**.

site. The docking experiment did not predict the formation of hydrogen bonds between Bcl-XL and either **21** or **22**. However, it should be noted that the flexibility of Bcl-XL may allow hydrogen bonding between the pyrazolyl group of **21** and **22** to a main-chain carbonyl as well as the side chain of Ser145 near the bottom of site 1, whereas in silico docking experiments performed with the rigid X-ray derived structure of Bcl-XL may be incapable of predicting these interactions.

In summary, based on available structural information of peptide and acylsulfonamide complexes with Bcl-XL, we have designed, synthesized, and conducted preliminary biological evaluations of pyrimidinylpiperazine based inhibitors of the Bcl-2 prosurvival protein family member Bcl-XL. Two series of pyrimidinylpiperazines were synthesized in few steps from readily available starting materials. The two series are distinguishable by the substituent on the pyrimidine ring which is either an ethyl or a benzyl group. The syntheses of both series are relatively straight forward and afford reasonable yields of final products. These pyrimidinylpiperazines showed promising activities on both the cytotoxic and Bcl-XL binding levels. These molecules conform to the standard Lipinski rules for druglikeness.[21,22] This new class of Bcl-XL inhibitors represent valuable new lead molecules for further development. Based on in silico docking experiments these molecules may take advantage of favorable shape complementary to the BH3 helix binding site, a matter which warrants further investigation.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2011.01.054.

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