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Screening of Protein−Protein Interaction Modulators *via* Sulfo-Click Kinetic Target-Guided Synthesis

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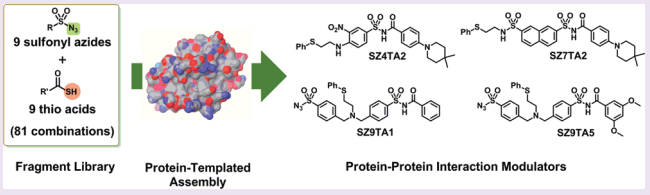
Supporting Information

ABSTRACT: Kinetic target-guided synthesis (TGS) and *in situ* click chemistry are among unconventional discovery strategies having the potential to streamline the development of protein−protein interaction modulators (PPIMs). In kinetic TGS and *in situ* click chemistry, the target is directly involved in the assembly of its own potent, bidentate ligand from a pool of reactive fragments. Herein, we report the use and validation of kinetic TGS based on the sulfo-click reaction between thio acids and sulfonyl azides as a screening and synthesis platform for the identification of high-quality PPIMs. Starting from a randomly designed library consisting of 9 thio acids and 9 sulfonyl azides leading to 81 potential acylsulfonamides, the target protein, Bcl-X_L, selectively assembled four PPIMs, acylsulfonamides **SZ4TA2**, **SZ7TA2**, **SZ9TA1**, and **SZ9TA5**, which have been shown to modulate Bcl-X_L/BH3 interactions. To further investigate the Bcl-X_L templation effect, control experiments were carried out using two mutants of Bcl-X_L. In one mutant, phenylalanine Phe131 and aspartic acid Asp133, which are critical for the BH3 domain binding, were substituted by alanines, while arginine Arg139, a residue identified to play a crucial role in the binding of **ABT-737**, a BH3 mimetic, was replaced by an alanine in the other mutant. Incubation of these mutants with the reactive fragments and subsequent LC/MS-SIM analysis confirmed that these building block combinations yield the corresponding acylsulfonamides at the BH3 binding site, the actual "hot spot" of Bcl-X_L. These results validate kinetic TGS using the sulfo-click reaction as a valuable tool for the straightforward identification of high-quality PPIMs.



Protein−protein interactions (PPIs) are central to a large number of vital biological processes and thus represent attractive targets for the development of novel therapies for a variety of diseases.[1−4] Although scientists have recognized the tremendous potential in targeting PPIs over the past two decades, the development of small molecules, which specifically modulate or disrupt a particular PPI, remains a challenging and risky undertaking.[1] Commonly, protein−protein interfaces are large and flat and lack deep cavities that might serve as good binding sites for small molecules.[5,6] Moreover, amino acids at the interfaces of PPIs are flexible and thus pose challenges at conducting computer-guided compound design.[7−9]

Although protein−protein interfaces bury 500−3000 Å^2 of total surface area, which exceeds the potential binding area of low-molecular-weight compounds,[10,11] Wells and co-workers demonstrated that only a fraction of the amino acid residues at the protein−protein interface contributes to the major portion of the binding free energy.[12−14] These key amino acids, defined as recognition patches or hot spots, therefore provide the theoretical and experimental evidence that PPIs can be disrupted or modulated by low-molecular-weight compounds. In the past 15 years, numerous approaches have been developed for the discovery of small molecules modulating or disrupting PPIs. Often, small molecule design is aimed at mimicking a peptide or a protein secondary structure in a truncated form.[15,16] Alternatively, fragment-based drug discovery strategies using biomolecular NMR, X-ray crystallography, or surface plasmon resonance (SPR) lead to the identification of fragments with good ligand efficiencies, which are further developed into potent protein−protein interaction modulators (PPIMs). Herein, we report the expansion and utilization of kinetic target-guided synthesis (TGS) as a screening platform for the identification of PPIMs.

In the past 2 decades, several TGS approaches have been described, in which the target biomolecule assembles its inhibitory ligand from a collection of reactive fragments. Depending on the nature of the assembly step, TGS approaches can be classified into (a) dynamic combinatorial chemistry (DCC), (b) reagent-accelerated TGS, and (c) kinetic TGS.[17−20] In dynamic combinatorial chemistry, the assembly process is reversible, whereas reagent-accelerated TGS uses building blocks, which combine in an irreversible fashion only in the presence of an external reagent or a catalyst upon binding to the biological target. In kinetic TGS, a biological target accelerates the irreversible covalent bond

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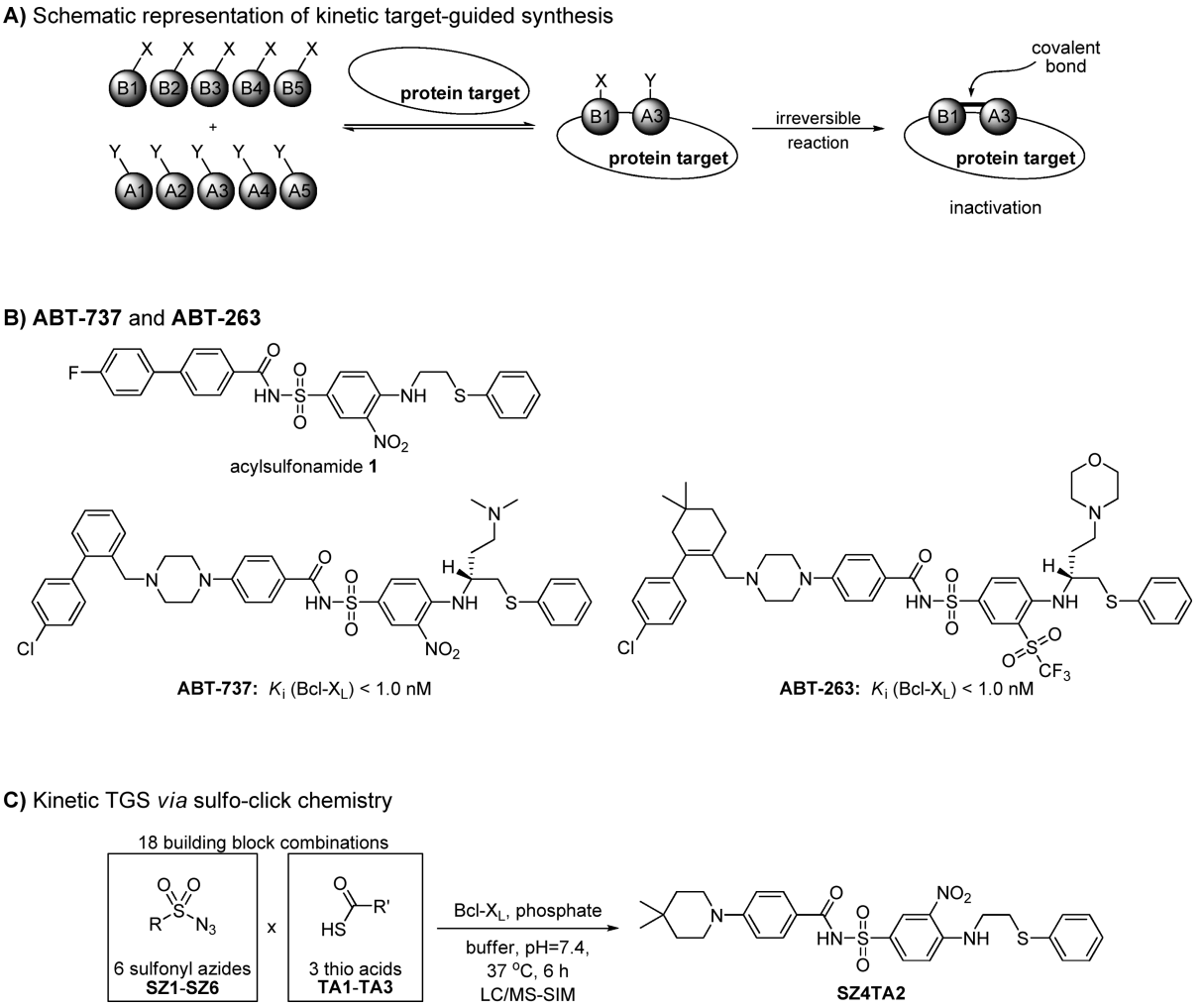

Figure 1. Kinetic TGS approach targeting PPIs. (A) TGS approaches are based on the principle that multidentate interactions between a ligand and a biological target are collectively much stronger than the corresponding monovalent interactions of each of the fragments.[60] Thus, a target-assembled compound most likely will have a stronger interaction with the biological target as compared to the individual building blocks.[60] In kinetic TGS, fragments decorated with complementary reactive groups are incubated with the target biomolecule. If two fragments reside simultaneously in close proximity in binding pockets of the target, the two reactive functionalities react with each other, forming a covalent linkage between the two fragments. (B) Acylsulfonamide **1**, **ABT-737**, and **ABT-263** compounds targeting Bcl-X_L. (C) Proof-of-concept study to demonstrate that the amidation between thio acids and sulfonyl azides is suited for kinetic TGS targeting PPIs.

formation only between complementary reacting fragments binding to adjacent binding sites of the target (Figure 1A). Kinetic TGS[16] and *in situ* click chemistry[17,18] have been exclusively applied for the identification of inhibitors of enzymatic targets with well-defined binding pockets. In a recent proof-of-concept study with the anti-apoptotic protein Bcl-X_L as the biological target, we demonstrated that kinetic TGS can also be used for the "rediscovery" of a PPIM previously reported by Abbott Laboratories starting from smaller fragments bearing a thio acid or a sulfonyl azide functional group.[20] Williams and co-workers described that the amidation reaction between thio acids and sulfonyl azides,[21,22] which in the meantime has been named the sulfo-click reaction,[23] proceeds in aqueous media.

The proteins of the Bcl-2 family have been validated as attractive PPI targets for cancer therapy.[24] The Bcl-2 family of proteins, which consists of both anti- and pro-apoptotic molecules, plays a pivotal role in the regulation of the intrinsic pathway of apoptosis. The anti-apoptotic Bcl-2 family proteins Bcl-2, Bcl-X_L, and Mcl-1 inhibit the release of certain pro-apoptotic factors from mitochondria. In contrast, pro-apoptotic Bcl-2 family members, which can be further separated into two subgroups, the multidomain BH1−3 proteins (*i.e.*, Bax and Bak) and the BH3-only proteins (*e.g.*, Bad, Bim, and Noxa), induce the release of mitochondrial apoptogenic molecules into the cytosol.[25,26] Evidence has been accumulated that the majority of human cancers overexpress the pro-survival Bcl-2 family proteins, which not only contribute to cancer progression by preventing normal cell turnover but also render cancer cells resistant to current cancer treatments.[27,28] Although there is a controversy over how anti-apoptotic Bcl-2 family proteins function,[29,30] it is generally accepted that apoptosis is initiated by the binding of pro-apoptotic BH3-only proteins to anti-apoptotic Bcl-2 family molecules in cancer cells. These interactions are mediated by the insertion of the BH3 domain of pro-death proteins into the hydrophobic groove on the surface of anti-apoptotic proteins Bcl-2, Bcl-X_L, or Mcl-1.[31,32] Therefore, small molecules that mimic the BH3 domains of pro-apoptotic Bcl-2 family proteins have potential as anticancer therapeutics.

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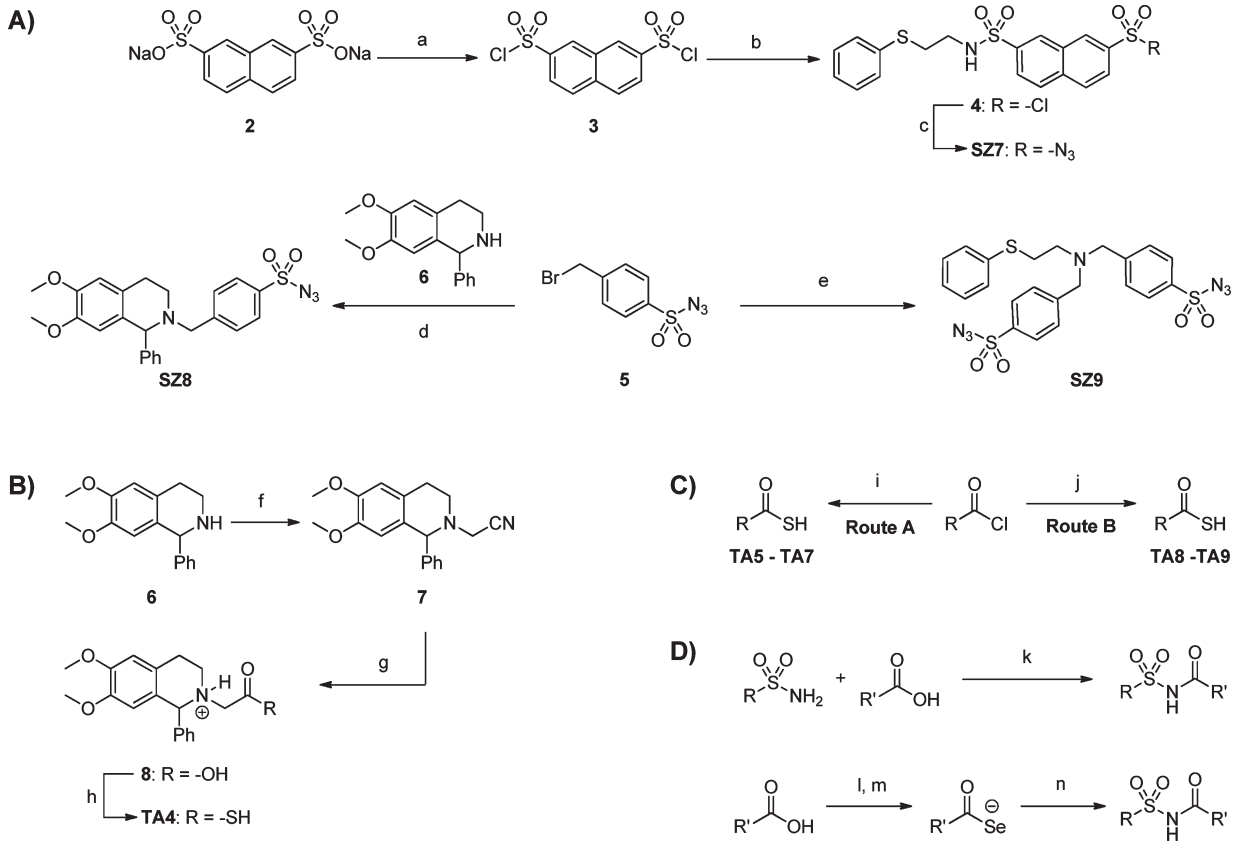

Figure 2. Synthesis of sulfonyl azides, thio acids, and acylsulfonamides. Reaction conditions: (a) $SOCl_2$, DMF, reflux, 2 h; (b) 2-(phenylthio)-ethanamine (0.5 equiv), K_2CO_3, $CHCl_3$, 12 h, RT; (c) NaN_3, acetone, H_2O, 0 °C, 3 h, 70% (over 3 steps); (d) K_2CO_3, CH_3CN/H_2O (9:1), 12 h, RT, 87%; (e) 2-(phenylthio)ethanamine (0.5 equiv), K_2CO_3, CH_3CN/H_2O (9:1), 12 h, RT, 60%; (f) ICH_2CN, K_2CO_3, CH_3CN/H_2O (10:1), 2 d, 60 °C, 79%; (g) 12 N HCl, 90 °C, 3 h, 66%; (h) (1) $(COCl)_2$, CH_2Cl_2, 0 °C to RT, 8 h, (2) dimethylthioformamide, H_2S, 15 min, 25%; (i) NaSH, acetone, H_2O, 2 h, RT; (j) NaSH, neat, 0 °C to RT, 1 h; (k) EDCI, DMAP, CH_2Cl_2, RT, 24−48 h; (l) $(CH_3)_2CHOCOCl$, *N*-methyl piperidine, THF, 0 °C, 30 min; (m) LiAlHSeH, THF, 0 °C, 30 min; (n) RSO_2N_3, THF, 0 °C to RT, 3 h.

Previously, Abbott Laboratories developed acylsulfonamide **1**, **ABT-737**, **ABT-263**, and other structurally related acylsulfonamides, which efficiently disrupt Bcl-X_L-Bad interaction (Figure 1B).[33−35] On the basis of these reports, we designed reactive fragments structurally related to **ABT-737** and **ABT-263** (**SZ1**−**SZ6** and **TA1**−**TA3**) and incubated these as binary fragment mixtures in the presence of Bcl-X_L (Figure 1C). Analysis of each incubation sample by liquid chromatography combined with mass spectrometry detection in the selected ion mode (LC/MS-SIM) showed that of all 18 possible products only compound **SZ4TA2**, which was developed by Abbott Laboratories, has been detected. In comparison, incubations of fragments in the absence of Bcl-X_L or in the presence of Bcl-X_L and various BH3-containing peptides failed to yield detectable amounts of acylsulfonamide products. In addition, IC_{50} inhibitory constants in the nanomolar range have been determined for **SZ4TA2**, while their corresponding thio acid or sulfonyl azide fragments did not show any inhibition up to 100 μM concentrations.

Herein, we successfully employed and validated the sulfo-click kinetic TGS approach as a straightforward yet reliable PPIM screening platform for the identification of Bcl-X_L-protein modulators. The design of kinetic TGS incubations with wildtype and mutant Bcl-X_L proteins provided an additional layer of confirmatory experiments for the delivery of high-quality PPIMs. Furthermore, experimental evidence has been accumulated indicating that kinetic TGS is a PPIM screening and synthesis method generating only active compounds.

RESULTS AND DISCUSSION

Screening of an Extended Reactive Fragment Library. The proof-of-concept study motivated us to investigate whether kinetic TGS is also successful at generating hit compounds that have not been previously reported. Two sublibraries of reactive fragments, one consisting of thio acids and the other of sulfonyl azides, have been synthesized. The thio acids were generated from the corresponding acid chlorides and sodium hydrosulfide, while the sulfonyl azides were prepared by alkylation of amines with 4-(bromomethyl)benzenesulfonyl azide (Figure 2A−C). A selection of acylsulfonamides has been synthesized mainly by (a) EDCI coupling of corresponding carboxylic acids and sulfonamides, or (b) the previously reported reaction between sulfonyl azides and selenocarboxylates that were generated from corresponding carboxylic acids and the selenating reagent LiAlHSeH (Figure 2D).[36]

The majority of the reactive fragments have been randomly selected, while a small fraction of the reactive fragments has been designed to be structurally related to **ABT-737** or **ABT-263**. Eighty-one binary mixtures containing one thio acid (**TA1**−**TA9**) and one sulfonyl azide (**SZ1**−**SZ9**) were incubated with the

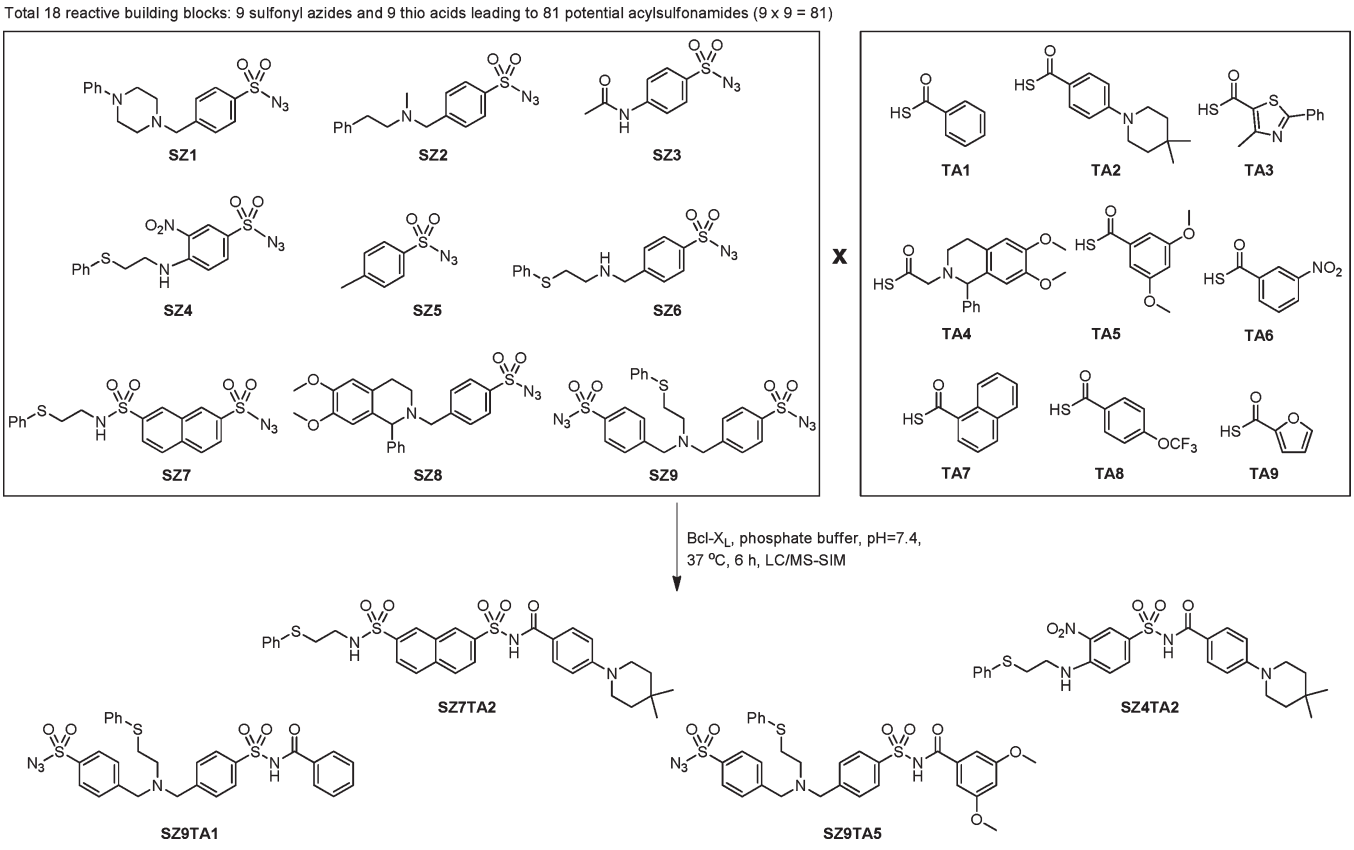

Figure 3. Kinetic TGS screening of Bcl-X_L *via* sulfo-click chemistry.

target protein Bcl-X_L for 6 h at 37 °C (Figure 3). In parallel, identical binary fragment mixtures were incubated in buffer without Bcl-X_L. Similar to *in situ* click chemistry,[17,18] all incubations were directly subjected to HPLC analysis with acylsulfonamide product detection by electrospray ionization in the positive selected ion mode (LC/MS-SIM).[37] Comparison of the LC/MS-SIM traces of identical fragment combinations with or without protein Bcl-X_L led to the identification of the previously reported fragment combination **SZ4TA2**[20] and three new combinations **SZ7TA2**, **SZ9TA1**, and **SZ9TA5** with increased amounts of acylsulfonamide products in the incubations containing Bcl-X_L (Figure 4A,B and Supporting Information).

Prior to synthesis of the new TGS hit compounds **SZ7TA2**, **SZ9TA1**, and **SZ9TA5**, control incubations with wildtype and mutant pro-apoptotic Bim BH3 peptides were conducted to assess whether the hit combinations assemble at the targeted binding sites of Bcl-X_L or randomly elsewhere on the protein surface (Figure 4C,D and Supporting Information). These control experiments with Bak BH3 peptide have been previously introduced to confirm the kinetic TGS assembly of compound **SZ4TA2**.[20] Wildtype Bim BH3 peptide (Bim sequence CEIWIAQELRRIGDEFNAYYAR), the natural Bcl-X_L ligand, outcompetes the reactive fragments for binding at the BH3 binding site of Bcl-X_L and thus suppresses the Bcl-X_L-templated assembly of acylsulfonamides **SZ7TA2**, **SZ9TA1**, and **SZ9TA5**. Contrarily, a mutant of the Bim BH3 peptide (mutant Bim sequence CEIWIAQEARRIGAEFNAYYAR) exhibits low affinity toward Bcl-X_L and therefore does not significantly affect the Bcl-X_L-templated assembly of **SZ7TA2**, **SZ9TA1**, and **SZ9TA5**. Since these co-incubations with wildtype and mutant BH3 peptides strongly suggest that the formation of acylsulfonamides **SZ7TA2**, **SZ9TA1**, and **SZ9TA5** takes place at the BH3 binding site of Bcl-X_L, compounds **SZ7TA2**, **SZ9TA1**, and **SZ9TA5** have been synthesized and subjected to LC/MS-SIM analysis. Comparison of the LC/MS-SIM traces of the Bcl-X_L-templated reactions with the ones of the synthetic compounds clearly confirmed that Bcl-X_L templates the formation of hit compounds **SZ7TA2**, **SZ9TA1**, and **SZ9TA5** (Figure 4E and Supporting Information).

Kinetic TGS with Mutant Bcl-X_L. Experiments were designed in which mutated Bcl-X_L proteins were incubated with reactive fragments. Alterations of the BH3 binding site directly affect the binding of reactive fragments **SZ4**, **SZ7**, **SZ9**, **TA1**, **TA2**, and **TA5** to the protein, which in turn will influence the rate of the protein-templated acylsulfonamide formation. The purpose of these mutant Bcl-X_L proteins is to expand the repertoire of controls with Bim BH3 peptides with complementary experiments indicating whether the TGS reaction occurs with the help of the target protein Bcl-X_L and specifically at the binding site of interest. The known mutant of Bcl-X_L, in which phenylalanine Phe131 and aspartic acid Asp133 have been substituted by alanines, has been prepared since it fails at interacting with Bak or Bim BH3 peptides.[38] In addition, a second mutant Bcl-X_L has been prepared, in which arginine Arg139 has been replaced by alanine. Arginine Arg139 has been identified to be a key residue interacting with **ABT-737** and analogues thereof.[33] As a proof-of-concept, incubations of the mutant Bcl-X_L with building blocks **SZ4** and **TA2** were first undertaken at various reactive fragment concentrations (Figures 5 and 6 and Supporting Information). In comparison to the incubation with wildtype Bcl-X_L, a reduction in the templation activity by approximately 40% or more has

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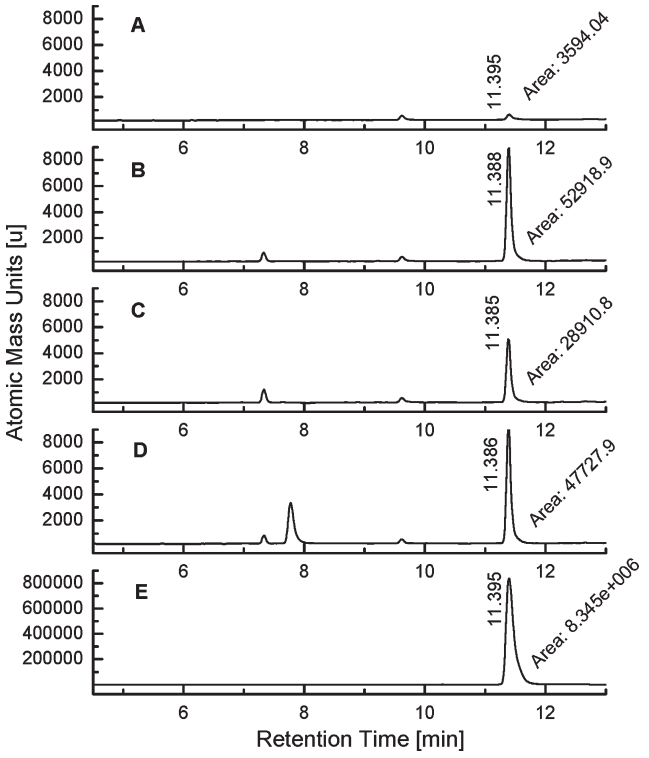

Figure 4. LC/MS-SIM analysis of kinetic TGS incubations with fragments **SZ7** and **TA2** targeting Bcl-X_L. The samples were incubated at 37 °C for 6 h and subjected to LC/MS-SIM analysis with gradient system 1 (see Supporting Information). (A) Incubation sample containing fragments **SZ7** and **TA2** in the absence of Bcl-X_L. (B) Incubation sample containing fragments **SZ7** and **TA2** in the presence of 2 μM Bcl-X_L. (C) Incubation sample containing fragments **SZ7** and **TA2** in the presence of 2 μM Bcl-X_L and 20 μM Bim BH3 peptide. (D) Incubation sample containing fragments **SZ7** and **TA2** in the presence of 2 μM Bcl-X_L and 20 μM mutant Bim BH3 peptide. (E) Synthetic **SZ7TA2** as the reference compound.

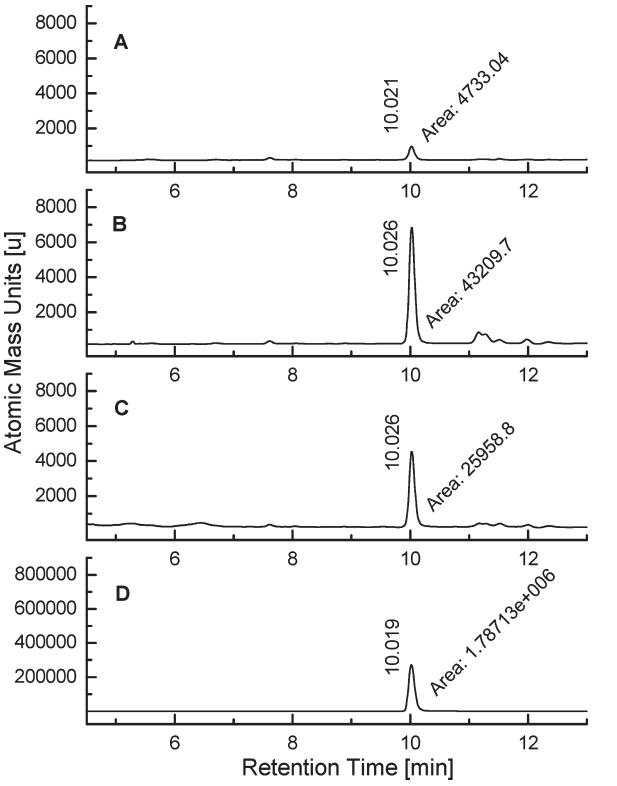

Figure 5. LC/MS-SIM analysis of kinetic TGS incubations with fragments **SZ4** and **TA2** targeting the wildtype and mutant of Bcl-X_L. The samples were incubated at 37 °C for 6 h and subjected to LC/MS-SIM analysis with gradient system 1 (see Supporting Information). (A) Incubation sample containing fragments **SZ4** and **TA2** in the absence of wildtype Bcl-X_L. (B) Incubation sample containing fragments **SZ4** and **TA2** in the presence of 2 μM wildtype Bcl-X_L. (C) Incubation sample containing fragments **SZ4** and **TA2** in the presence of 2 μM single mutant ^{R139A}Bcl-X_L. (D) Synthetic **SZ4TA2** as the reference compound.

been observed in both mutant Bcl-X_L-templated reactions (Table 1). This observation can be explained by closer examination of a reported NMR-structure of Bcl-X_L complexed with acylsulfonamide **1**, whose structure is closely related to the kinetic TGS product **SZ4TA2**.[33] Comparison of the location of Phe131 and Asp133 with the position of compound **1** in the wildtype Bcl-X_L binding site reveals that the residues Phe131 and Asp133, although important for the binding to Bak or Bim BH3 peptides, are relatively distant from the acylsulfonamide **1**, whereas Arg139 appears to be closer to compound **1**. Surprisingly, mutant ^{R139A}Bcl-X_L displays a slightly increased templation reaction in comparison to ^{F131A,D133A}Bcl-X_L. Conformational changes induced by seemingly distant amino acid residues are difficult to trace and may probably influence the templation effect observed during the incubations with wildtype and mutant Bcl-X_L proteins.

For TGS hit combinations **SZ7TA2**, **SZ9TA1**, and **SZ9TA5**, confirmatory experiments have been conducted with single mutant ^{R139A}Bcl-X_L only, since the preparation of double mutant ^{F131A,D133A}Bcl-X_L has been cumbersome. Similar to the incubations of fragments **SZ4** and **TA2**, experiments with the mutant protein leading to acylsulfonamides **SZ7TA2**, **SZ9TA1**, and **SZ9TA5** displayed a reduction in acylsulfonamide formation compared to the incubations with wildtype Bcl-X_L. These experiments suggest that the acylsulfonamide genesis occurs in proximity to key amino acid residue Arg139.

PPIM Activity of Kinetic TGS Hits and Additional Acylsulfonamides. The kinetic TGS hits were subjected to dose−response studies to obtain IC_{50}s and to investigate if the hit compounds are also modulating or disrupting the interaction between Bcl-X_L and a native BH3 peptide ligand. Previously, Abbott Laboratories determined by their assay that **SZ4TA2** is a good PPIM with a K_i constant of 19 nM.[34,35] Abbott determined the dissociation constants by a competitive fluorescence polarization assay using a fluorescein-labeled Bad BH3 peptide. In order to precisely compare the inhibitory properties of our kinetic TGS hits with the compounds reported by Abbott, we decided to perform binding studies by a fluorescence polarization assay implemented in our laboratories, which uses GST-Bcl-X_L and fluorescein-labeled Bak BH3 peptide. Consistently, compound **SZ4TA2** has been validated by our assay as a Bcl-X_L inhibitor against Bak BH3 with an IC_{50} constant of 106 nM (Table 2). Kinetic TGS hit compounds **SZ7TA2**, **SZ9TA1**, and **SZ9TA5** showed IC_{50}s in the low micromolar range (Figure 3 and Supporting Information). Taken together, these results indicate that the hit compounds **SZ4TA2**, **SZ7TA2**, **SZ9TA1**, and **SZ9TA5** identified through the kinetic TGS screening are indeed respectable ligands of the biological target, which

underscores the utility of kinetic TGS as a valuable approach to PPIM discovery.

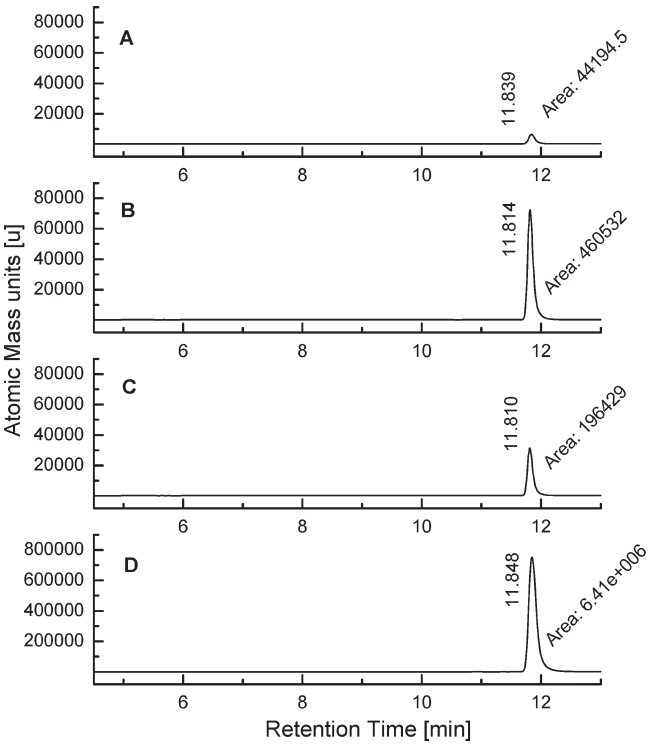

Figure 6. LC/MS-SIM analysis of kinetic TGS incubations with fragments **SZ4** and **TA2** targeting the wildtype and double mutant of Bcl-X_L. The samples were incubated at 37 °C for 6 h and subjected to LC/MS-SIM analysis with gradient system 2 (see Supporting Information). (A) Incubation sample containing fragments **SZ4** and **TA2** in the absence of wildtype Bcl-X_L. (B) Incubation sample containing fragments **SZ4** and **TA2** in the presence of 2 μM wildtype Bcl-X_L. (C) Incubation sample containing fragments **SZ4** and **TA2** in the presence of 2 μM double mutant ^{F131A,D133A}Bcl-X_L. (D) Synthetic **SZ4TA2** as the reference compound.

To assess whether the kinetic TGS hits are more potent than acylsulfonamides, which were not identified in the kinetic TGS screening, 33 randomly selected acylsulfonamides were synthesized. All compounds, as well as TGS hit compounds **SZ4TA2**, **SZ7TA2**, **SZ9TA1**, and **SZ9TA5**, were tested at a 50 μM concentration for PPI disruption in the Bcl-X_L/Bak BH3 fluorescence polarization assay. The 37 acylsulfonamides tested correspond to 45.7% of the 81-member library. Strikingly, the four kinetic TGS hits were the most potent compounds tested, disrupting the Bcl-X_L/BH3 interaction with 60% inhibition or more, while the randomly selected acylsulfonamides demonstrated an average of 15% inhibition (Table 3). Only four of the 33 randomly selected acylsulfonamides demonstrated moderate inhibition (35−45%). In contrast, all reactive fragments **SZ1**−**SZ9** and **TA1**−**TA9** have been tested in the fluorescence polarization assay at 100 μM concentration, and less than 5% inhibition was detected. These measurements indicate that the dissociation constants for the corresponding reactive building blocks **SZ1**−**SZ9** and **TA1**−**TA9** have to be higher than 100 μM. These important results suggest that the amidation reaction between thio acids and sulfonyl azides is suitable for kinetic TGS using building blocks displaying weak binding affinities. In addition, this study strongly suggests that the kinetic TGS screening identified the more active members of the library of potential acylsulfonamides arising from reactive fragments **SZ1**−**SZ9** and **TA1**−**TA9**.

Discussion. Generally, cell-permeable small modulators of PPIs have been considered to be desirable tools with great implications for drug discovery and development.[3,4]

Table 2. PPIM Activity of Kinetic TGS Hit Compounds

compound	IC_{50}	K_i
SZ4TA2	106 ± 12 nM	37.5 ± 5.0 nM
SZ7TA2	28.4 ± 3.5 μM	11.5 ± 1.4 μM
SZ9TA1	28.7 ± 4.1 μM	11.6 ± 1.6 μM
SZ9TA5	36.0 ± 2.5 μM	14.6 ± 1.0 μM

Table 1. Kinetic TGS Incubations[a]

	fragment combinations							
	SZ4TA2		**SZ7TA2**		**SZ9TA1**		**SZ9TA5**	
incubation	peak area	% signal	peak area	% signal	peak area	% signal	peak area	% signal
buffer alone	26,794	7.4	3,594	6.8	313	35.3	466	10.9
WT Bcl-X_L	363,187	100.0	52,920	100.0	887	100.0	4,275	100.0
WT Bcl-X_L and WT Bak BH3	59,437	16.3	nd	nd	nd	nd	nd	nd
WT Bcl-X_L and mutant Bak BH3	181,156	49.8	nd	nd	nd	nd	nd	nd
WT Bcl-X_L and WT Bim BH3	51,773	14.3	28,911	54.6	552	62.2	944	22.1
WT Bcl-X_L and mutant Bim BH3	217,813	59.9	47,728	90.2	761	85.8	2,557	59.8
buffer alone	44,195	9.6	nd	nd	nd	nd	nd	nd
WT Bcl-X_L	460,532	100.0	nd	nd	nd	nd	nd	nd
^{F131A,D133A}Bcl-X_L	196,429	42.7	nd	nd	nd	nd	nd	nd
buffer alone	4,733	11.0	2,046	7.2	939	25.0	726	11.4
WT Bcl-X_L	43,210	100.0	28,600	100.0	3,750	100.0	6,370	100.0
^{R139A}Bcl-X_L	25,959	60.1	16,965	59.3	2,637	70.3	4,406	69.2

[a] nd = not determined; WT = wildtype

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Table 3. Percentage Inhibition Displayed by an Acylsulfonamide at 50 μM[a]

Fragments	SZ1	SZ2	SZ3	SZ4	SZ5	SZ6	SZ7	SZ8	SZ9
TA1	nd	2	0	14	29	nd	nd	19	80
TA2	nd	8	nd	100	28	26	76	nd	38
TA3	6	7	nd	nd	nd	nd	nd	30	22
TA4	nd	25	nd	nd	nd	nd	nd	8	nd
TA5	5	nd	nd	nd	0	nd	15	11	60
TA6	4	nd	0	nd	0	nd	20	nd	nd
TA7	nd	nd	0	nd	nd	nd	47	30	45
TA8	nd	nd	0	nd	nd	nd	nd	38	nd
TA9	3	nd	0	nd	1	nd	nd	24	nd

[a] nd = not determined

Nevertheless, reliable yet straightforward techniques or approaches for the development of potent and effective PPIMs are currently unavailable. Over the past 15 years, a variety of fragment-based lead discovery approaches have been developed and successfully applied for the development of potent PPIMs.[39−41] These approaches are commonly based on the detection of fragments binding to the target protein followed by the study of their binding to the protein target at atomic level resolution using X-ray crystallography or NMR spectroscopy. The initial hits are further optimized *via* fragment growing, in which fragments are extended into identified binding sites step-by-step, or *via* fragment linking, in which fragments identified to bind to adjacent binding sites are covalently linked together.[41−44] Even though fragment-based lead discovery strategies have been very successful for the development of PPIMs, they are mainly limited by two constraints. Detection and quantification of fragment binding requires specially designed methodology due to the weak binding typically observed for fragments. Furthermore, the optimization of fragments into potent and selective compounds is not straightforward and not rapidly achievable, even though structural information is available.[43,45] For example, though high quality NMR structures were available, the development of Bcl-X_L PPIMs by Abbott[33,34] required several design iterations, and the preparation and testing of more than 1000 compounds in order to yield **ABT-737** and **ABT-263**.[46] Furthermore, of the very first design consisting of 21 different structures containing the structural motifs of the initial fragments identified by NMR, most compounds bound to Bcl-X_L with a dissociation constant greater than 10 μM.[35] Thus, though the hit compounds **SZ7TA2**, **SZ9TA1**, and **SZ9TA5** display IC_{50} constants of 28−37 μM in the Bak BH3 fluorescence polarization assay, the herein reported kinetic TGS approach suggests that the high-quality PPIMs will be identified early on in the screening process. This outcome is consistent with previously reported kinetic TGS studies, in which the enzyme carbonic anhydrase II preferably accelerates the formation of the better inhibitory compounds from a pool of reactive fragments.[47,48] Other kinetic TGS examples using exclusively *in situ* click chemistry also suggest that the triazoles generated in the protein-templated reactions are the more potent inhibitors.[37,48−54]

Recently, fragment-based discovery strategies have been reported that involve the protein target directly selecting and assembling its own inhibitory compounds from a pool of reactive fragments. These approaches, also termed *in situ* click chemistry or kinetic TGS approaches,[16,18] were conceptually described in the 1980s[55] and are still relatively unexplored compared to dynamic combinatorial chemistry. Thus far, kinetic TGS has mainly been applied to the identification of potent enzyme inhibitors. Nevertheless, the herein reported kinetic TGS offers an attractive approach to PPIM lead discovery because it allows the protein to select and combine building blocks that fit best into its binding sites, thus assembling larger compounds.[16,18] The screening method can be as simple as determining whether the PPIM product has been formed in a given test mixture. This is especially advantageous over a conventional high-throughput screening of difficult targets such as protein interfaces requiring cumbersome and time-consuming experiments to confirm whether screening hits are true or false positives.

Finally, considering that the flexible nature of protein interfaces complicates the development of PPIMs by conventional means, kinetic TGS has the potential to target the protein in a conformation that is short-lived, undetectable, or easily missed with present techniques. A small number of *in situ* click chemistry approaches targeting enzymatic systems lead to the identification of triazole inhibitors stabilizing the protein in an unprecedented and less abundant conformation.[56−58] Thus, we speculate that the herein reported sulfo-click chemistry kinetic TGS approach provides medicinal chemists a straightforward search strategy to stabilize conformations of dynamic protein targets such as PPIs.

Conclusions. Herein, we demonstrate that the sulfo-click kinetic TGS approach exhibits great promise in fragment-based PPIM discovery since it combines synthesis and screening of libraries of low-molecular-weight PPIMs into a single step. Samples containing the protein target Bcl-X_L and reacting fragments leading to 81 structurally different acylsulfonamides have been incubated and analyzed by LC/MS-SIM for acylsulfonamide formation. Of the 81 possible fragment combinations, only combinations **SZ4TA2**, **SZ7TA2**, **SZ9TA1**, and **SZ9TA5** yielded acylsulfonamides in the Bcl-X_L-templated reactions. Control incubations with the four hit fragment combinations, in which the BH3 binding site of the wildtype Bcl-X_L has been competitively occupied by a Bim BH3 peptide, generated decreased amounts of acylsulfonamides. Furthermore, control incubations with mutants ^{R139A}Bcl-X_L or ^{F131A,D133A}Bcl-X_L, in which amino acid residues at the BH3 binding site have been replaced by alanines, also failed at generating the hit acylsulfonamides, suggesting that the protein-templated assembly of **SZ4TA2**, **SZ7TA2**, **SZ9TA1**, and **SZ9TA5** occurs at the desired BH3 binding site of Bcl-X_L. Subsequent testing of synthesized kinetic TGS hit acylsulfonamides in a fluorescence-based competitive binding assay demonstrated that the kinetic TGS hit compounds indeed display PPIM activity. These findings have been supported by a set of 33 additional acylsulfonamides randomly selected from the 81-member library, which have been shown to fail at demonstrating potent PPIM activity in the fluorescence-based competitive binding assay. These results provide a general test case for the sulfo-click kinetic TGS approach to generate hits targeting the proteins of the Bcl-2

family and further validate the kinetic TGS approach to be suitable for PPIM discovery. In contrast to conventional screening approaches, experimental data suggests that PPIM screening *via* kinetic TGS reduces the number of false positives, cutting down the number of screening hits to be validated in confirmatory assays. We speculate that the herein reported PPIM discovery strategy for the family of the Bcl-2 proteins is general and can easily be implemented to lead development targeting other PPIs such as MDM2/p53, IAP/caspase, and others.[1,4,39]

METHODS

Synthesis of Selected Compounds. The synthesis of reactive fragments and acylsulfonamides is reported in the Supporting Information.

Expression and Purification of Wildtype and Mutant Bcl-X_L Fusion Proteins. The protocols are reported in the Supporting Information.

General Protocol for Incubations of Bcl-X_L with Reactive Fragments. In a 96-well plate, one thio acid building block (1 μL of a 2 mM solution in methanol) and one sulfonyl azide building block (1 μL of a 2 mM solution in methanol) were added to a solution of Bcl-X_L (98 μL of a 2 μM Bcl-X_L solution in buffer (58 mM Na_2HPO_4, 17 mM NaH_2PO_4, 68 mM NaCl, 1 mM NaN_3, pH = 7.40)). The 96-well plate was sealed and incubated at 37 °C for 6 h. The incubation samples were then subjected to liquid chromatography combined with mass spectrometry analysis in the selected ion mode (LC/MS-SIM, Zorbax SB-C18 preceded by a Phenomenex C18 guard column, electrospray ionization, and mass spectrometric detection in the positive selected ion mode, tuned to the expected molecular mass of the product). The TGS hit compound was identified by the mass and the retention time. As a control, identical building block combinations were incubated in buffer without Bcl-X_L and subjected to LC/MS-SIM analysis. Comparison of the LC/MS-SIM chromatograms of these control incubations with the chromatograms of the Bcl-X_L-containing incubations allows us to determine whether the protein is templating the corresponding amidation reaction. Furthermore, synthetically prepared acylsulfonamide was subjected to LC/MS-SIM analysis, and the retention time was compared with the one identified in the Bcl-X_L containing incubation.

Fluorescence Polarization-Based Competitive Binding Assay. The detailed protocol to conduct fluorescence polarization-based competitive binding assays has been previously reported.[20]

ASSOCIATED CONTENT

Supporting Information. Synthetic procedures, LC/MS-SIM traces, ^1H and ^{13}C NMR spectra and determination of IC_{50} values. This material is available free of charge *via* the Internet at http://pubs.acs.org.

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