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Synthesis of 4-(2-Phenylhydrazono)-1-(4-phenylthiazol-2-yl)-1*H*-pyrazol-5(4*H*)-one Compounds and Characterization of Their Affinities to Anti-apoptotic Bcl-2 Family Proteins

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Organic compounds containing the thiazol-2-yl-1*H*-pyrazol-5(4*H*)-one moiety are known to be associated with versatile pharmacological applications. In this study, we describe the methods for preparing 4-(2-phenylhydrazono)-1-(4-phenylthiazol-2-yl)-1*H*-pyrazol-5(4*H*)-one compounds. A set of 26 compounds were synthesized with overall yields ranging between 37%—92%. They were tested in a fluorescence polarization-based binding assay against three anti-apoptotic Bcl-2 family proteins, including Bcl-x_L, Bcl-2, and Mcl-1. Our results indicate that this class of compounds are not effective inhibitors of these anti-apoptotic Bcl-2 family proteins. Their apoptosis-inducing effects are possibly due to BAX activation as suggested by Gavathiotis *et al.* in their recent study. However, other possibilities should not be ignored. In addition, a crystal structure obtained by us reveals that the exocyclic double bond in the molecular structure of this class of compounds is in the (*Z*)-configuration.

Keywords apoptosis inducer, BAX activation, Bcl-2 inhibition, protein-protein interaction, small-molecule inhibitor

Introduction

Organic compounds containing the thiazol-2-yl-1*H*-pyrazol-5(4*H*)-one moiety (Figure 1), obtained either from natural resources or chemical synthesis, are known to be associated with versatile pharmacological applications.[1-6] In our previous studies, we synthesized this class of compounds and screened them using MTT assays on several tumor cell lines, including A549, HeLa and MDA-MB-231 cells. Indeed, some of them exhibited obvious cytotoxicity (CG_{50} = 1—20 μmol•L^{-1}) on

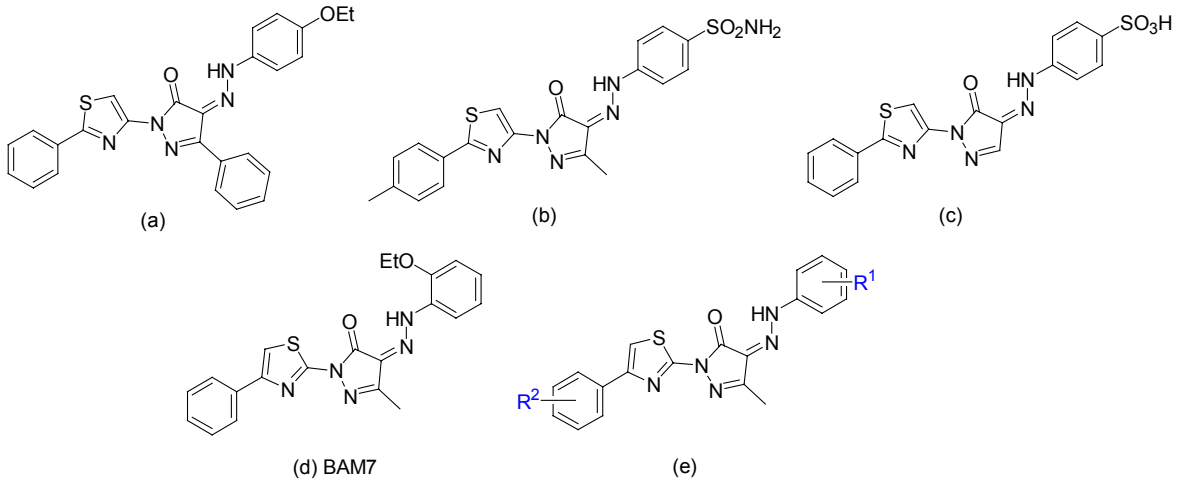

Figure 1 Some known compounds containing the thiazol-2-yl-1*H*-pyrazol-5(4*H*)-one moiety, which were reported as (a) causing revert cholesterol accumulation in Niemann-Pick C cells,[4] (b) treating neurological and neurodegenerative diseases,[5] (c) shp-2 inhibitor,[6] (d) apoptosis inducer by BAX activation[8] and (e) apoptosis inducer described in our previous study.[7]

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these tumor cells, and our preliminary studies indicated that this class of compounds induced apoptosis in tumor cell lines.[7] However, the exact molecular mechanism of the apoptosis-inducing effects of this class of compounds remains unknown.

Recently, Gavathiotis *et al*.[8] reported an interesting study regarding the regulation of Bcl-2 family proteins with small-molecule compounds. Bcl-2 family proteins are key regulators of the apoptotic pathway. Anti-apoptotic members, such as Bcl-2, Bcl-x_L, and Mcl-1, sequester the Bcl-2 homology 3 (BH3) death domains of pro-apoptotic members, such as BAX, to maintain cell survival.[9-11] The BH3-binding groove on anti-apoptotic Bcl-2 family proteins has been considered as target for developing small-molecule apoptosis inducers as potential anti-cancer therapies.[12] A number of inhibitors of Bcl-2 family proteins have been successfully developed during the past decade or so.[13-30] But Gavathiotis *et al*. attempted to find compounds that can mediate direct BAX activation to induce apoptosis. A total of 750000 molecules were examined by means of computational screening. Finally, 100 of them were selected and tested. Based on the results of NMR and biochemical analyses, they demonstrated that one compound engaged the BAX trigger site and promoted the functional oligomerization of BAX. The molecule was observed to induce cell death in a BAX-dependent manner.

Gavathiotis' study drew our attention since the most active compound described by them, *i.e*. BAM7 in Figure 1, belongs to the same class of thiazol-2-yl-1*H*-pyrazol-5(4*H*)-one compounds tested in our previous studies. Their results provide hints to understanding the molecular mechanism of this class of compounds. Since both activation of BAX and inhibition of anti-apoptotic Bcl-2 family proteins will induce apoptosis through the mitochondrial pathway,[9-11] it is also necessary to verify if this class of compounds actually interact with anti-apoptotic Bcl-2 family proteins. This type of verification, however, was not systematically performed by Gavathiotis *et al*. in their study.

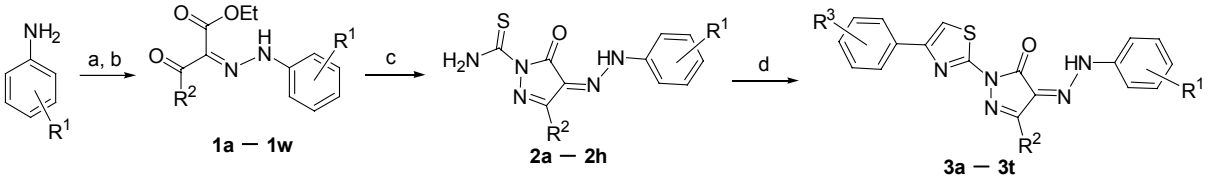
In this study, we describe a set of efficient synthetic methods for preparing this class of compounds. All of the 26 obtained compounds, including BAM7, were tested in a fluorescence polarization-based *in vitro* binding assay to measure their binding affinities to three anti-apoptotic Bcl-2 family proteins, *i.e*. Bcl-x_L, Bcl-2, and Mcl-1. Our results indicate that this class of compounds do not bind effectively with any of these three Bcl-2 family proteins. Our study thus provides an additional support to Gavathiotis' conclusion that this class of compounds induce apoptosis through BAX activation rather than Bcl-2 inhibition.

Experimental

Organic synthesis

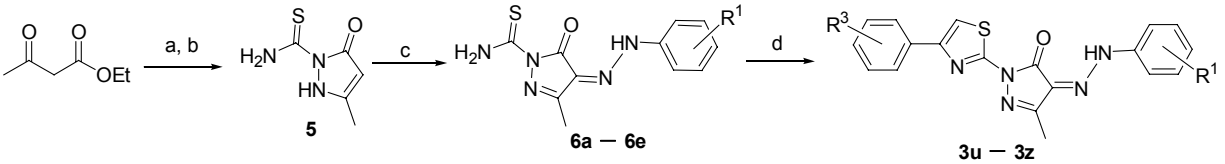
The target compounds **3a**—**3t** and **3u**—**3z** were prepared with the synthetic methods illustrated in Schemes 1 and 2, respectively. In brief, target compounds were synthesized by three steps: Firstly, substituted ethyl acetoacetate underwent coupling reaction with aromatic diazonium salts in aqueous solution to yield **1**. Then, **1** was cyclized with thiosemicarbazide under reflux in HOAc to give **2**. Finally, heated in dioxane, **2** and phenacyl bromide underwent the Hantzsch thiazole reaction to yield compounds **3a**—**3t** (Scheme 1).[31,32] When R^1 was CH_2OH, hydroxylmethyl group would esterify with HOAc under the reaction condition. Therefore, the synthetic route of compounds **3u**—**3z** was altered as: Ethyl acetoacetate was treated with thiosemicarbazide in 5% HCl solution, and was then heated with NaOAc in EtOH to obtain ***5***, which existed in its enamine form. Then, **5** was coupled with diazonium salts to produce **6**. The final step was also the cyclization reaction with phenacyl bromide to obtain the target compounds **3u**—**3z** (Scheme 2).[33]

Scheme 1 General synthetic methods for obtaining hydrazono-thiazolyl-pyrazolone compounds



Reagents and conditions: (a) $NaNO_2$, HCl, 0—5 ℃; (b) substituted ethyl acetoacetate, NaOAc, EtOH, r.t., 1 h; (c) thiosemicarbazide, HOAc, reflux, 4 h; (d) *α*-bromoacetophenone, dioxane, reflux, 0.5 h.

Scheme 2 Revised synthetic methods for obtaining hydrazono-thiazolyl-pyrazolone derivatives **6a**—**6e**



Reagents and conditions: (a) thiosemicarbazide, 5% HCl, H_2O, r.t., 1 h; (b) EtOAc, EtOH, reflux, 4 h; (c) substituted aniline, $NaNO_2$, HCl, 0—5 ℃; (d) *α*-bromoacetophenone, dioxane, reflux, 0.5 h.

The synthetic methods for preparing the necessary starting material or intermediates as well as spectral data of compounds **3a**—**3z** are given in the Supporting Information.

Crystal structure determination

Growth of single crystals was attempted for **3y**. Single crystals in good quality were obtained using the following condition. The compound sample (10 mg) was dissolved in DCM (0.5 mL) and *n*-hexane (2.0 mL) in a 5 mL sample bottle. The mixture was then transferred into a 60 mL reagent bottle containing 20 mL *n*-hexane. The solution was allowed to vaporize for several days to obtain the crystals.

X-ray crystal diffraction data were collected on a Bruker Apex II CCD diffractometer operating at 50 kV and 30 mA using Mo Kα radiation (λ=0.71073 Å) at 293 K. Main parameters of this crystal structure are: $C_{21}H_{19}N_5O_2S$, M_r=405.48 g/mol, crystal size 0.31 mm ×0.12 mm×0.09 mm. Triclinic, space group *P*-1, a=5.0025(6) Å, b=12.4830(15) Å, c=16.0296(19) Å, α=75.651(3)°, β=85.409(3)°, γ=81.677(3)°, V=958.5(2) Å^3, Z=2, D_c=1.045 g•cm^{-3}, λ=0.71073 Å, μ(Mo Kα) =0.198 mm^{-1}. This crystal structure has been deposited in the Cambridge Crystal Data Center (access number: 927666).

***In vitro* binding assay**

Fluorescence polarization (FP)-based assay was employed in our study to measure the binding affinities of small-molecule compounds to three human anti-apoptotic Bcl-2 family proteins, *i.e.* Bcl-x_L, Bcl-2, and Mcl-1. In this binding assay, a 26-residue peptide derived from the BH3 domain on the Bid protein with 5-carboxyfluorescein (5-FAM) on the N-terminus, *i.e.* 5-FAM-QEDIIRNIARHLAQVGDSM-DRSIPPG, was used as the fluorescence tracer. Our dose-dependent saturation experiments determined that this Bid-BH3 peptide bound to Bcl-x_L with K_d=41 nmol•L^{-1}, to Bcl-2 with K_d=89 nmol•L^{-1}, and to Mcl-1 with K_d=29 nmol•L^{-1}.

In each measurement, the Bid-BH3 peptide at a total concentration of 10 nmol•L^{-1} was incubated with the protein first. Total concentration of the protein was set to five times of the K_d value of the fluorescence tracer, *i.e.* 205 nmol•L^{-1} in the case of Bcl-x_L, 445 nmol•L^{-1} in the case of Bcl-2, and 145 nmol•L^{-1} in the case of Mcl-1. Competitive binding of a given compound was characterized quantitatively by monitoring the changes in FP signals upon the addition of the compound at a series of doses. As a validation, ABT-263, a well-known Bcl-2 inhibitor was tested in our binding assay as a positive control. Our measured inhibition constants (K_i) of ABT-263 against Bcl-x_L and Bcl-2 were lower than 1 nmol•L^{-1}, which was consistent with the data reported in literature.[13,14] Our measured K_i value of ABT-263 against Mcl-1 was 1.6 μmol•L^{-1}, which was also basically consistent with the reported data of 0.55 μmol•L^{-1}.[14]

In this study, each compound was tested against all three target proteins (Bcl-x_L, Bcl-2 and Mcl-1) at three different concentrations, *i.e.* 1, 10 and 50 μmol•L^{-1}. At each concentration, the average FP value of three parallel measurements was used to compute inhibition ratio using the following equation.

$$\text{Inhibition}=\frac{mP_{\max}-mP_{\text{test}}}{mP_{\max}-mP_{\text{NC}}}\times 100\%$$

Here, $mP_{\max}$ refers to the FP signal detected when the protein was incubated with the Bid-BH3 peptide, mP_{NC} refers to the FP signal detected from the negative control, *i.e.* the Bid-BH3 peptide alone, and mP_{test} refers to the FP signal detected when the compound under test was added at a certain concentration. Because most compounds tested in this study did not exhibit significant binding affinities to three target proteins, no additional attempt was made to determine accurate IC_{50} values or inhibition constants for these compounds.

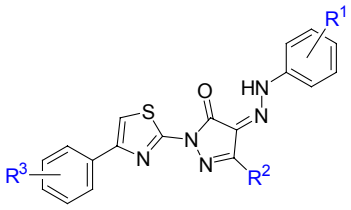
Inhibition rates measured at 50 μmol•L^{-1} for all compounds are summarized in Table 1. The full results can be found in the Supporting Information. Detailed descriptions of the methods used for expression and purification of three proteins, the FP-based assay, and data processing can be found in one of our recent publications.[26]

Results and Discussion

In this study, a total of 26 compounds were obtained (Table 1). These compounds were synthesized by the methods illustrated in Scheme 1 and Scheme 2. Different substituent groups were installed at three sites on the framework to explore a preliminary structure-activity relationship of this class of compounds. As for R^1, some small polar groups were considered to improve solubility since the framework of this class of compounds is apparently not very soluble. As for R^2, three hydrophobic groups of different sizes, *i.e.* methyl, isopropyl, and phenyl groups were chosen by considering synthetic feasibility. R^3 was basically a substituted phenyl or naphthyl group, which resembles the chemical structures of the same class of compounds investigated by other researchers (Figure 1).

In this study, we obtained the crystal structure of **3y**, *i.e.* compound BAM7 described in Gavathiotis' study.[8] The crystal structure is shown in Figure 2. One can see that this molecule has a rather flat shape, in which the four 5-member and 6-member rings are aligned basically in one plane. The torsion angle between atoms C(5)-N(4)-N(5)-C(8) is −179.8° indicating that this "linker" part also locates on the same plane. Since the other compounds synthesized by us (Table 1) are different from **3y** only in terminal substituent groups, it is reasonable to assume that their three-dimensional structures are similar to that of **3y**.

Table 1 Inhibitory activities of compounds **3a**—**3z** on three anti-apoptotic Bcl-2 family proteins



Compd. ID	Symbol	R^1	R^2	R^3	Yield%	Inhibition rate at 50 μmol•L^{-1}/%			Ref.[a]
						Bcl-x_L	Bcl-2	Mcl-1	
BCL-SSC-01	**3a**	3-OCH_3	Me	3-OMe-C_6H_4	74	17	14	7	
BCL-SSC-02	**3b**	2-CH_2OH	Me	3-OMe-C_6H_4	92	35	43	23	[7]
BCL-SSC-03	**3c**	3-CH_2OH	Me	3-OMe-C_6H_4	75	30	39	–12	[7]
BCL-SSC-04	**3d**	2-OCH_3	*i*-Pr	3-OMe-C_6H_4	72	–19	14	–32	
BCL-SSC-05	**3e**	3-CO_2CH_3	*i*-Pr	3-OMe-C_6H_4	83	–3	–6	25	
BCL-SSC-06	**3f**	3-OCH_3	*i*-Pr	3-OMe-C_6H_4	64	6	–6	30	
BCL-SSC-07	**3g**	3-NO_2	*i*-Pr	3-OMe-C_6H_4	89	–2	31	39	
BCL-SSC-08	**3h**	3-CO_2CH_3	Ph	3-OMe-C_6H_4	70	16	4	35	
BCL-SSC-09	**3i**	2-CO_2CH_3	Ph	3-OMe-C_6H_4	72	9	4	30	
BCL-SSC-10	**3j**	2-NO_2	Ph	3-OMe-C_6H_4	85	35	37	30	
BCL-SSC-11	**3k**	2-OCH_3	Ph	3-OMe-C_6H_4	55	9	17	34	
BCL-SSC-12	**3l**	3-OCH_3	Ph	3-OMe-C_6H_4	59	5	41	46	
BCL-SSC-13	**3m**	3-NO_2	Ph	3-OMe-C_6H_4	37	19	39	47	
BCL-SSC-14	**3n**	2-OCH_3	Me	2-Naphthyl	68	–13	–9	9	
BCL-SSC-15	**3o**	3-CH_2OH	Me	2-Naphthyl	79	7	20	28	
BCL-SSC-16	**3p**	2-CO_2CH_3	Me	2-Naphthyl	81	12	14	43	
BCL-SSC-17	**3q**	3-OCH_3	*i*-Pr	2-Naphthyl	64	–3	0	13	
BCL-SSC-18	**3r**	3-CO_2CH_3	*i*-Pr	2-Naphthyl	85	7	2	34	
BCL-SSC-19	**3s**	3-CH_2OH	Me	3,4-$(OMe)_2$-C_6H_3	43	29	9	19	
BCL-SSC-20	**3t**	3-CH_2OAc	Me	3,4-$(OMe)_2$-C_6H_3	41	–9	–9	18	
BCL-SSC-21	**3u**	4-SO_2NH_2	Me	4-OMe-C_6H_4	63	23	49	56	[7]
BCL-SSC-22	**3v**	3-COOH	Me	4-OMe-C_6H_4	67	21	43	53	[7]
BCL-SSC-23	**3w**	3-Br	Me	4-OMe-C_6H_4	58	0	25	16	
BCL-SSC-24	**3x**	3-Br	Me	3,4-$(OMe)_2$-C_6H_3	54	25	31	38	
BCL-SSC-25	**3y**	2-OEt	Me	Ph	62	25	31	38	[8]
BCL-SSC-26	**3z**	2-OEt	Me	4-OMe-C_6H_4	56	14	34	44	

[a] A few compounds were reported in our previous study (ref. [7]) or Gavathiotis' study (ref. [8]). All of the other compounds are new compounds that have not been reported in literature before.

A notable feature in the three-dimensional structure of **3y** is the intramolecular hydrogen bonds formed between N(5)—H···O(1) and N(5)—H···O(2) (Figure 2). This bifurcate hydrogen bond network further immobilizes the 1*H*-pyrazol-5(4*H*)-one ring, the linker part, and the substituent phenyl ring in one common plane. Due to the existence of this hydrogen bond, the C(5)=N(4) double bond is of the (*Z*)-configuration. Note that Gavathiotis *et al*. derived a binding mode of BAM-7 (**3y**) in complex with BAX through molecular modeling, in which the C(5) = N(4) double bond was of the (*E*)-configuration.[8]

It is reasonable to expect that the (*Z*)-configuration of **3y** is energetically favored. In order to prove this, we employed density functional theory computations to estimate the energy difference between (*Z*)-**3y** and (*E*)-**3y**. Computations were performed using the

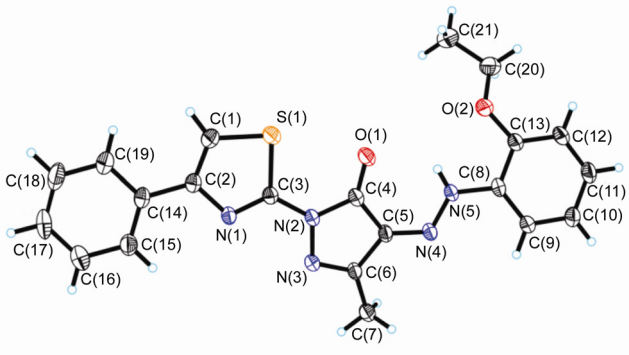


Figure 2 Crystal structure of **3y** (BAM-7). Formula $C_{21}H_{19}N_5O_2S$, molecular weight 405.4729. Cambridge Crystallographic Data Center (CDCC) deposition number: 927666.

Gaussian 09 package. Geometry optimization of both structures was performed at the B3LYP/6-311＋＋G(d,p) level. Both optimized structures were proved to be minima on the potential energy surface by vibrational frequency analysis (Figure 3). Single-point energies were then computed for each structure in gas phase as well as in water solvent using a PCM solvation model. The computed energy difference between (*Z*)-**3y** and (*E*)-**3y** is 8.79 kcal/mol in gas phase and 5.03 kcal/mol in water, respectively. This energy gap is significant even in water. Accordingly, it is reasonable to expect that BAM-7 binds to BAX in its low-energy (*Z*)-configuration rather than the (*E*)-configuration. In other words, binding of BAM-7 to BAX is not likely to compensate the energy penalty needed for breaking the intramolecular hydrogen bond in the (*Z*)-configuration and converting this molecule into the (*E*)-configuration. Thus, we believe that the binding mode of BAM-7 to BAX shown in Gavathiotis' study is not correct.

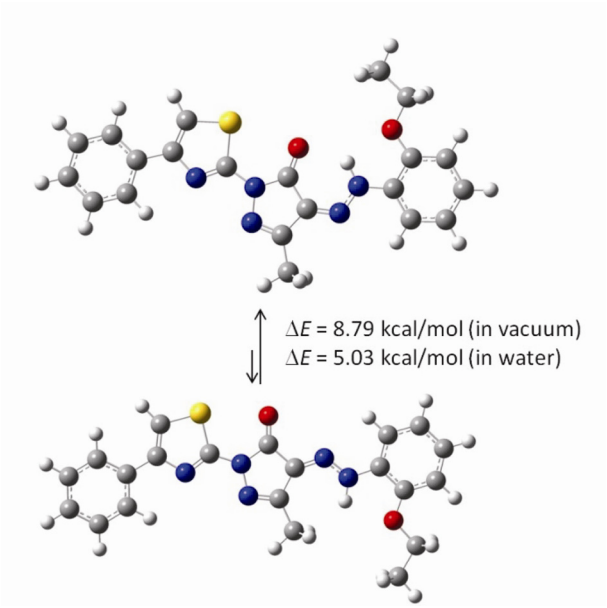


Figure 3 Difference in the structures and energies of (*Z*)-**3y** (top) and (*E*)-**3y** (bottom). Energies were computed at the B3LYP/6-311＋＋G(d,p) level.

Binding affinities of this class of compounds to three anti-apoptotic Bcl-2 family proteins measured in our study are also summarized in Table 1. One can see that about half of these compounds have weak interactions with three Bcl-2 proteins. For example, compounds **3u** and **3v** exhibited inhibition rates around 50% at the highest concentration tested in our study (50 μmol•L^{-1}). Other compounds basically do not interact with three Bcl-2 proteins. We thus conclude that this class of compounds, if the molecular framework is not changed significantly, are not effective inhibitors of anti-apoptotic Bcl-2 family proteins. Their apoptosis-inducing effects should be attributed to other molecular mechanisms, possibly through BAX activation as reported by Gavathiotis *et al*. in their recent study.[8]

But other possible apoptosis-inducing mechanisms for this class of compounds should not be completely ruled out. As implied by their versatile biological effects reported by different researchers (Figure 1), we speculate that this class of compounds actually has multiple molecular targets in cell. In fact, apoptosis is controlled by such a complex biological network. Many factors have contributions to this process. For example, some Bcl-2 inhibitors were originally identified as mechanism-based apoptosis inducers, such as HA14-1 [15] and gossypol[16,17]. But it was revealed later in more sophisticated studies that these compounds did not function solely as BH3 mimetics since their cytotoxic activity was not dependent on Bax or Bak.[34] Gavathiotis *et al*. demonstrated nicely with Bax and/or Bak knockout mouse embryo fibroblasts (MEFs) that BAM7 triggers *in vitro* BAX oligomerization, BAX-mediated pore formation and BAX-dependent cell death.[8] In fact, they also tested 16 other analogs of BAM7 and found that those analogs did not function in the same manner. Thus, the role of this class of compounds as apoptosis inducers still needs to be interpreted with extreme care.

Conclusions

In this study, we have synthesized a set of 26 4-(2-phenylhydrazono)-1-(4-phenylthiazol-2-yl)-1*H*-pyrazol-5(4*H*)-one compounds. The crystal structure of one selected compound clearly reveals that the exocyclic double bond on the core moiety is in the (*Z*)-configuration due to the formation of intramolecular hydrogen bonds. All obtained compounds were tested against three anti-apoptotic Bcl-2 family proteins in binding assay. Our results indicate that generally speaking, they are not effective Bcl-2 inhibitors. Thus, their apoptosis-inducing effects are possibly due to BAX activation as suggested by Gavathiotis *et al*. rather than Bcl-2 inhibition. However, as implied by the versatile biological effects of this class of compounds, the molecular mechanism of their apoptosis-inducing effects is perhaps more sophisticated than solely BAX activation.

Acknowledgements

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