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# Investigation of novel pyrazole carboxamides as new apoptosis inducers on neuronal cells in *Helicoverpa zea*

Yuanhang Ren, Na Yang, Ying Yue, Hong Jin, Ke Tao *, Taiping Hou

*Key Laboratory of Bio-Resource and Eco-environment of Ministry of Education, College of Life Sciences, Sichuan University, Chengdu 610064, China*

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ABSTRACT

Novel pyrazole carboxamides with a diarylamine-modified scaffold were modified based on the bixafen (Bayer) fungicide, which has excellent activity against *Rhizoctonia solani, Rhizoctonia cerealis and Sclerotinia sclerotiorum.* To discover the potential insecticidal activity of these novel pyrazole carboxamides, the present study explored their possible cytoactivity on the insect neuronal cells (RP-HzVNC-AW1) in *Helicoverpa zea*. The preliminary bioassays showed that some of the target compounds exhibited good cytoactivity against AW1 cells. Among them, compounds **a5** and **b4**–**b7** showed good activity *in vitro* with $IC_{50}$ values of 11.28, 10.46, 9.04, 11.72 and 12.19 μM, respectively. Notably, the $IC_{50}$ value of compound **b5** was better than 11.81 μM for fipronil. We subsequently attempted to illustrate the mechanism of **b5**. Intracellular biochemical assays showed that **b5** induced AW1 cell apoptosis with a decrease in themitochondrial membrane potential, as well as a significantly increased intracellular calcium ion concentration and caspase-3 activity. A significant decrease in Bcl-2 levels and a marked augmentation of cytochrome-c and Bax were also detected. These results indicate that a mitochondrially dependent intrinsic pathway contributes to compound **b5**-induced apoptosis in AW1 cells. This study suggests that **b5** may act as a potential insecticide that can be used for further optimization.



## 1. Introduction

Agricultural crop losses caused by pests and plant diseases represent a worldwide issue and often threat crop production around the world.[1–4] Developing unique and efficacious chemicals for controlling pests and disease is one of the goals of researchers. The series of novel pyrazole carboxamides with diarylamine-modified scaffolds were designed and synthesized by our group.[5] These compounds were modified based on a commercial fungicide bixafen (Bayer) and replaced the biphenyl group with diarylamines. Subsequently, *in vitro* and *in vivo* bioassays with the new compounds exhibited good antifungal activity against *Rhizoctonia solani, Rhizoctonia cerealis* and *Sclerotinia sclerotiorum.*[5] However, more attention was paid on their fungicidal activity, and few studies were reported on the impact of pyrazole carboxamide compounds on their cytoactivity and insecticidal activity. In our previous work, we screened various compounds against insect neuronal cells (RP-HzVNC-AW1) derived from *Helicoverpa zea* for cytoactivity.[6,7] Among the compounds, we found that pyrazole carboxamide compounds can significantly inhibit AW1 cell proliferation. Thus far, only a very small number of reports have shown cytoactivity of pyrazole carboxamide.[8–10]

Apoptosis is ubiquitous in animals and is essential in every life stage.[11] Apoptosis can be triggered through two main pathways that are known as the extrinsic (death receptor-dependent) and intrinsic (mitochondrial-dependent, endoplasmic reticulum-dependent) pathways.[12] In the intrinsic pathway, the release of death factors into the cytosol from the mitochondria and the regulation of Bcl-2 family proteins contributes to the activation of apoptosis. For instance, once cytochrome-c, the main death factor, is released into the cytosol, it activates caspase-9, which subsequently activates the effector caspase known as caspase-3. Finally, the activated effector caspases irreversibly lead to cell death.[12,13] Bcl-2 family proteins play a vital role in the apoptotic process to regulate the release of cytochrome-c. Most researchers believe that Bax/Bcl-2 are the key factors that regulate apoptosis, and they determine the prognosis of apoptosis and tumour markers.[14–16] Meanwhile, intracellular calcium ($[Ca^{2+}]_i$), which is a secondary messenger, plays a critical role in regulating cells apoptosis.[17–19] The rise in $[Ca^{2+}]_i$ also results in the release of cytochrome-c and cytochrome-a activation of caspase-12 and caspase-3.[20]

Pyrazole derivative-induced mammalian cell death has been shown to be associated with the apoptotic and autophagic pathways,[10] although the physiological and mechanistic significance

* Corresponding author.
*E-mail address:* taoke@scu.edu.cn (K. Tao).

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of these changes have never been clearly defined in insect cells. In our study, we tried to screen out several compounds with high cytoactivity and determine the mechanism of action of these compounds in AW1 cells. This finding would not only supply evidence for identifying the apoptotic pathway used by insect cells but would also make pyrazole carboxamide a new potential insecticide.

In this paper, we screened several compounds that exhibited good cytoactivity using the MTT assay. We then used different methods to show its ability to induce apoptosis in AW1 cells. Our results indicate that **b5**, one of the pyrazole carboxamides, can downregulate the mitochondrial membrane potential, induce cytochrome-c release, activate caspase-3, upregulate the concentration of intracellular calcium ions and induce the expression of Bax / Bcl-2. Taken together, this evidence shows that AW1 cells undergo caspase-dependent mitochondrial apoptosis.

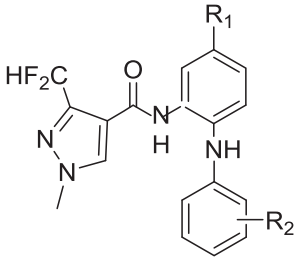
## 2. Results and discussion

### 2.1. Cytotoxic activities against AW1 cells

The pyrazole carboxamide compounds are often the focus of its fungicidal activity. However, to uncover the potential insecticidal activities of these novel pyrazole carboxamides, the present study aimed to explore their possible cytoactivity on insect neuronal cells (AW1) of *Helicoverpa zea*. As shown in Table 1, the cytotoxicity of pyrazole carboxamide compounds on AW1 was assessed at different concentrations (100, 50, 25, 12.5 and 6.25 μM) for 48 h. In general, most of the compounds showed good potency against AW1 cells. At the concentration of 100 μM, 10 compounds showed strong cytotoxic effects (above 90%) on AW1 cells, and we chose to focus on fipronil (98.62%). The results also indicate that compounds **a5** and **b4**–**b7** inhibited AW1 cells in a dose-dependent manner (Table 1). Of note, Table 2 shows that the $IC_{50}$ values of **a5** and **b4**–**b7** were 11.28, 10.46, 9.04, 11.72 and 12.19 μM, respectively, while the $IC_{50}$ was close to 11.81 μM for fipronil (Table 2). These results strongly suggested that the pyrazole carboxamide compounds can effectively inhibit AW1 proliferation.

Cell-based high throughput assays can quickly investigate the lead compound in insecticides and provide a novel angle for studying the toxicology and mechanism of action of new insecticide candidates at the molecular and cellular level. A great majority of these studies on the activity and toxicity of insecticides has been carried out using insect cell lines derived from the ovaries and embryos of pests.[21] However, to our knowledge, the target of most insecticides is the insect nervous system,[22] and there are few studies that have reported the use of the insect nerve cell line in the field of pesticide discovery. Therefore, in our study, an insect nerve cell line (AW1) was used to investigate the cytoactivity of a series of novel pyrazole carboxamide compounds. This finding will con-

**Table 1**
The cytoactivity of pyrazole carboxamide compounds and fipronil against AW1.




| Compounds | | | AW1 cytoactive (%) at a concentration of (μM) | | | | |
|---|---|---|---|---|---|---|---|
| No. | $R_1$ | $R_2$ | 100 | 50 | 25 | 12.5 | 6.25 |
| **a1** | F | 2-F | 85.28 | 24.85 | – | – | – |
| **a2** | F | 2, 4-$F_2$ | 82.45 | * | – | – | – |
| **a3** | F | 3, 4-$F_2$ | 87.49 | 73.50 | 47.48 | 30.48 | * |
| **a4** | F | 2-Cl | 82.18 | 26.79 | – | – | – |
| **a5** | F | 3-Cl | 96.51 | 89.26 | 81.80 | 41.51 | 29.23 |
| **a6** | F | 4-Cl | 95.00 | 26.85 | – | – | – |
| **a7** | F | 4-Br | 96.01 | 94.61 | 46.51 | 19.63 | – |
| **a8** | F | 4-$CH_3$ | 70.61 | 22.81 | – | – | – |
| **a9** | F | 4-$OCH_3$ | 86.27 | 14.53 | – | – | – |
| **b1** | Cl | H | 65.47 | 21.49 | – | – | – |
| **b2** | Cl | 2-F | 50.29 | 28.82 | – | – | – |
| **b3** | Cl | 4-F | 89.51 | 74.39 | 46.96 | 25.50 | – |
| **b4** | Cl | 3, 4-$F_2$ | 98.92 | 95.10 | 80.33 | 32.53 | 25.56 |
| **b5** | Cl | 4-Cl | 97.26 | 85.70 | 79.81 | 42.39 | 32.49 |
| **b6** | Cl | 4-Br | 98.43 | 95.96 | 80.24 | 45.15 | 34.84 |
| **b7** | Cl | 4-$CH_3$ | 97.77 | 96.18 | 75.17 | 33.35 | 20.70 |
| **b8** | Cl | 4-$OCH_3$ | 95.01 | 49.12 | * | – | – |
| **c1** | H | H | 26.18 | 15.63 | – | – | – |
| **c2** | H | 2-F | 53.71 | * | – | – | – |
| **c3** | H | 4-F | 60.38 | 39.98 | * | – | – |
| **c4** | H | 2, 4-$F_2$ | 50.17 | 28.44 | – | – | – |
| **c5** | H | 3-Cl | 70.89 | 26.94 | – | – | – |
| **c6** | H | 4-Cl | 98.87 | 98.69 | 27.14 | – | – |
| **c7** | H | 2, 4-$Cl_2$ | 50.17 | 28.44 | – | – | – |
| **c8** | H | 4-Br | 60.82 | 24.16 | – | – | – |
| **c9** | H | 4-$CH_3$ | 93.28 | 91.04 | 35.80 | * | – |
| **c10** | H | 4-$OCH_3$ | 61.05 | 32.02 | – | – | – |
| Fipronil | | | 98.62 | 85.73 | 72.91 | 61.48 | 25.61 |

Values are the mean of five replicates. "–" represent non-test, "*" represent no significant activity.

**Table 2**
$IC_{50}$ values for compounds **a5 and b4–b7** and fipronil against AW1.

| Compounds | *y = a + bx* | R | $IC_{50}$ (μM) |
|---|---|---|---|
| **a5** | y = 2.8305 + 2.0613x | 0.9815 | 11.28 |
| **b4** | y = 2.7016 + 2.2546x | 0.9796 | 10.46 |
| **b5** | y = 3.0738 + 2.0146x | 0.9613 | 9.04 |
| **b6** | y = 2.5267 + 2.3141x | 0.9629 | 11.72 |
| **b7** | y = 2.4075 + 2.3875x | 0.9602 | 12.19 |
| Fipronil | y = 2.6913 + 2.1532x | 0.9774 | 11.81 |

tribute to a better understanding of the relationship between compounds and their targets.

### 2.2. Effects of b5 on AW1 cell apoptosis

To further study the insecticidal mechanism of these novel compounds, we examined compound **b5**, which had the highest cytotoxicity, as a representative compound. The effect of **b5** on apoptosis in AW1 cells was determined by flow cytometry. After treatment with 10, 50, 100 μM of **b5** for 24 h, an increasing number of cells began to shrink, float from the bottom of the plates (data not shown) and undergo fragmentation. We further demonstrated the occurrence of apoptosis by Annexin V/PI staining methods (Fig. 1A). The flow cytometry assay showed that the ratio of apoptotic cells increased from 11.03 ± 0.5% in 0.1% DMSO-treated cells to 48.5 ± 1.8%, 58.9 ± 1.2%, 69.90 ± 2.5% in **b5**-treated cells (Fig. 1B), a finding that indicated that **b5** could potentially induce apoptosis in AW1 cells.

### 2.3. Effects of b5 on AW1 cell mitochondrial membrane potential

The mitochondrial membrane potential (ΔΨm) is caused by the asymmetric distribution of protons and other ions in the two sides of mitochondrial membrane. ΔΨm regulates mitochondrial membrane selectivity and permeability for various substances to maintain normal mitochondrial morphology and function.[23] Loss of the mitochondrial membrane potential (ΔΨm) is a marker of mitochondrial function and is often associated with apoptosis.[24] Our results indicated that the ΔΨm in the treated cells decreased gradually as the concentration of **b5** increased from 10 to 100 μM. The peak values of Rh 123 fluorescence decreased by 30.69 ± 2.3% ($^{*}P < 0.05$) after treatment with 10 μM of **b5** for 24 h (Fig. 2A, B). Furthermore, as illustrated in Fig. 2C, a significant decrease of 38.73 ± 1.4% ($^{**}P < 0.01$) and 49.52 ± 0.9% ($^{**}P < 0.01$) in the mitochondrial membrane potential (ΔΨm) was observed after treatment with 50 and 100 μM of **b5** for 24 h, respectively.

### 2.4. Effects of compounds on the level of apoptotic proteins

The Bcl-2 family, which consists of both the Bax pro-apoptotic proteins and the Bcl-2 anti-apoptotic proteins, regulates the permeability of the mitochondrial membrane, cytochrome c release and activation of caspase-3.[25] Studies have indicated that under various types of apoptotic stimulation, the overexpression of the Bcl-2 anti-apoptotic protein can prevent cytochrome c release.[26] Western blotting showed altered levels of cytochrome c, Bcl-2, and Bax in AW1 cells treated with different concentrations of **b5** for 24 h (Fig. 3). In the control group (0.1% DMSO treated), we observed high expression levels of Bcl-2 and only a slight expression of Bax. As shown in Fig. 3, Bcl-2 was downregulated, and Bax was upregulated in a dose-dependent manner after **b5** treatment. Cytochrome-c is a protein that is anchored in the inner mitochondrial membrane by its association with anionic phospholipids.[27] It is an essential factor in the mitochondrially mediated apoptotic pathway and plays a key role in apoptosis.[28] Extracted cytosolic fractions of AW1 cells treated with different concentrations of **b5** were also analysed by Western blot. As shown in Fig. 3, cytochrome-c levels increased with **b5** treatment in a dose-dependent manner.

The activation of caspases is a key feature of apoptosis. Therefore, the pro-apoptotic protein caspase-3 was studied. AW1 cells were exposed to 10, 50, and 100 μM of compound **b5** for 0, 12 and 24 h, respectively, and the resultant activities of caspase-3 were determined. As shown in Fig. 4, after treatment with **b5**, the activity of caspase-3 increased significantly after 12 h and 24 h. The apoptotic signal pathways typically involve caspase activation and the caspase cascade.[29,30] The initial caspase (caspase-8 or caspase-9) is activated by the extrinsic inducer. Finally, the executioner caspase, such as caspase-3, is activated to result in substrate degradation and induce apoptosis.

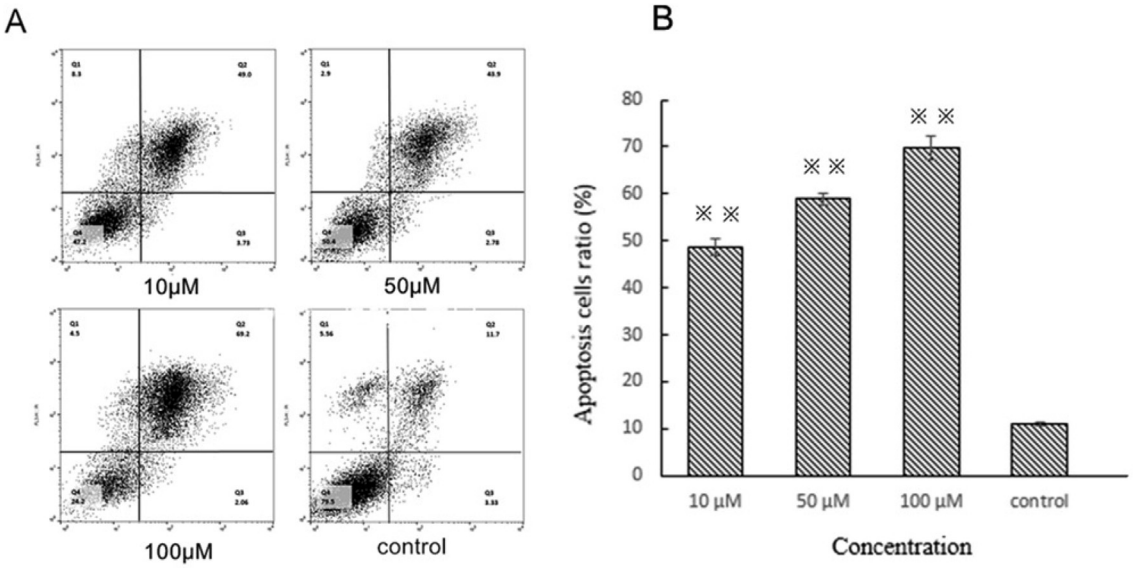



**Fig. 1.** The apoptotic effect of **b5** on AW1 cells. Cells were stained with Annexin V-EGFP and propidium iodide (PI) after treatment with 10, 50, and 100 μM compounds for 24 h (A). The ratio of apoptotic cells (percentage of Q2 plus Q3) after treatment with 10, 50, and 100 μM **b5** for 24 h (B) $^{*}P \leq 0.05$ and $^{**}P \leq 0.01$ vs. the negative control.

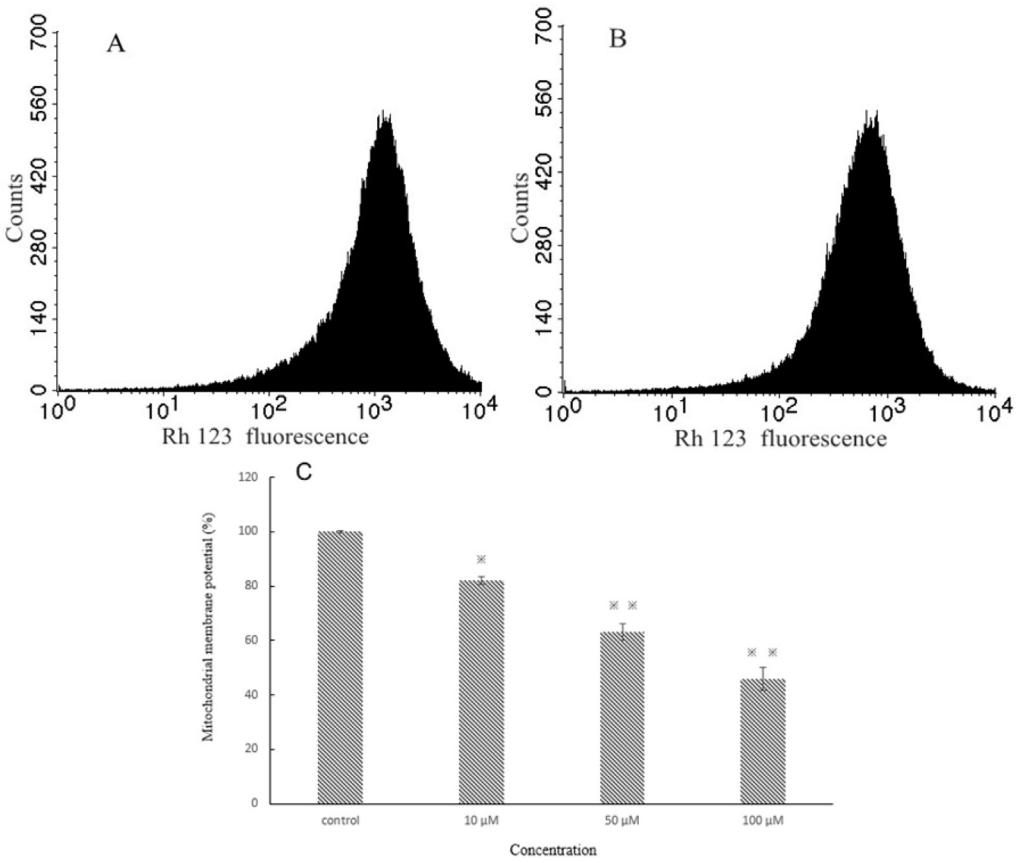



**Fig. 2.** Effect of **b5** on the mitochondrial membrane potential. A: control cells; B: cells treated with 10 μM of **b5**; C: cells treated with 10, 50, and 100 μM of **b5** for 24 h $^{*}P \leq 0.05$, $^{**}P \leq 0.01$ vs. negative control.

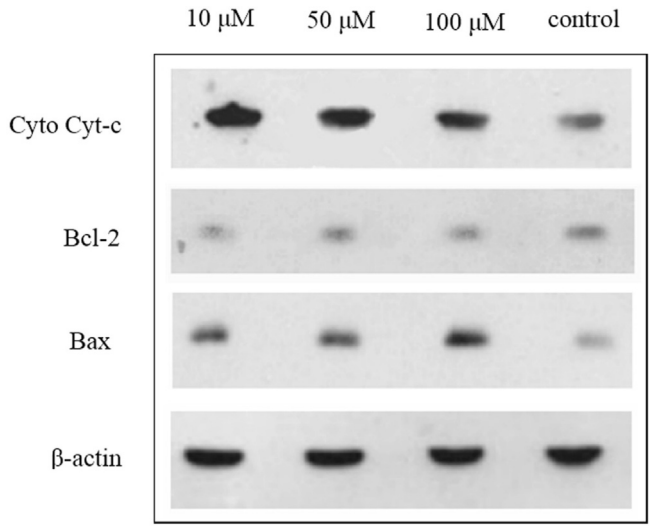



**Fig. 3.** The expression analysis of apoptotic proteins in AW1 cells. AW1 cells were incubated with 10, 50, and 100 μM of **b5** for 24 h. The expression levels of Cytc, Bcl-2 and Bax were determined by Western blotting.

Apoptosis is known to rely on two major pathways, the cell death receptor-mediated extrinsic pathway and the mitochondrially mediated intrinsic pathway.[31] The two most important characteristics of the mitochondrially mediated apoptotic pathway are the loss of ΔΨm and the release of cytochrome-c. In fact, loss of the mitochondrial membrane potential (ΔΨm) is considered the earliest event in the apoptotic cascade and is one of the specific signs of apoptosis.[28] Moreover, many apoptotic responses are initiated by the delivery of cytochrome-c from the mitochondria, leading to the activation of caspase-3 and subsequent apoptosis.[31] Our data clearly show that **b5** could induce ΔΨm collapse and reduce the expression of Bcl-2 in AW1 cells, which may increase the release of cytochrome-c from the mitochondria into the cytoplasm and activate caspase-3.

### *2.5. Effects of b5 on the intracellular calcium concentration in AW1 cells*

The effects of **b5** on the intracellular calcium ($[Ca^{2+}]_i$) concentration in AW1 cells were studied by detecting the calcium concentration after the neurons had been dyed with fluo-3 AM. Fig. 5 show the change in $[Ca^{2+}]_i$ versus the recording time when AW1 was treated with different concentrations of **b5** in the absence of extracellular calcium. Fluorescence values were expressed as F/F0, with F0 being the resting (or baseline) fluorescence and F being the change in fluorescence from baseline after application of the drug. The results indicated that the calcium concentrations increased in the presence of **b5** in a dose-dependent manner. Application of 10, 50, and 100 μM of compound **b5** resulted in an increase in $[Ca^{2+}]_i$ to 129.80 ± 1.2%, 121.27 ± 2.8%, and 136.38 ± 4.2% of the initial value. The results also indicate that the elevation of $[Ca^{2+}]_i$ was attributed to the release of calcium from the endoplasmic reticulum, and compound **b5** could activate calcium release channels on the ER.

Intracellular calcium can regulate cell cycle progression, proliferation, apoptosis and many other cellular processes. Recent research has shown that in the intrinsic pathway as a response

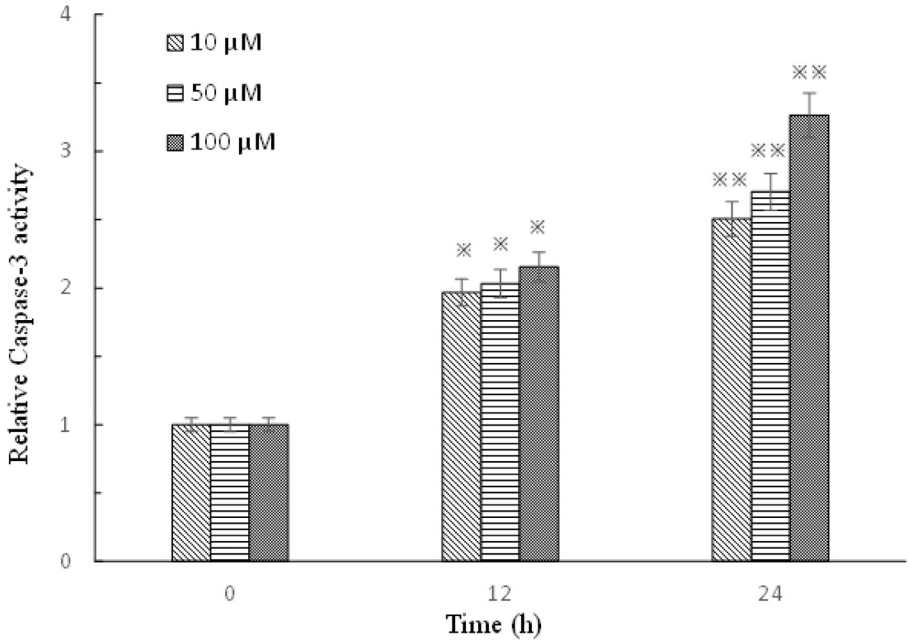



**Fig. 4.** Effects of **b5** on the activity of caspase-3 in AW1 cells. Cells were treated with the b5 compound at 10, 50, and 100 μM for 12 h and 24 h. $^{*}P \leq 0.05$, $^{**}P \leq 0.01$ vs. the negative control.

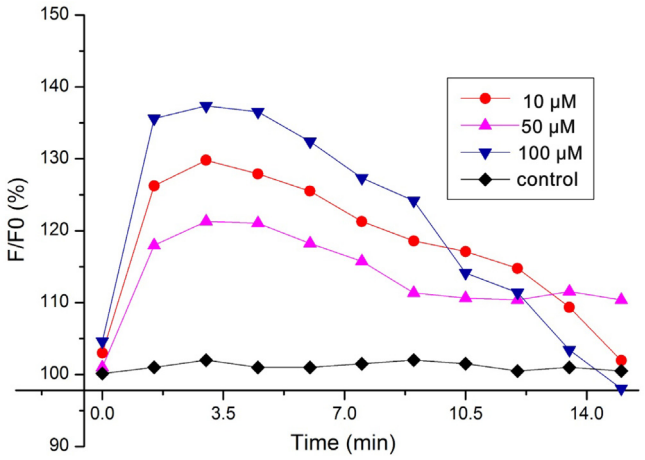



**Fig. 5.** The effects of **b5** on $[Ca^{2+}]_i$ in AW1 cells. The fluo-3 AM dye was added to cells along with 10, 50 or 100 μM of b5 when extracellular $Ca^{2+}$ was absent.

to endogenous and exogenous factors, calcium participated in this process and played a vital role.[32] Researchers have noted that the $Ca^{2+}$-dependent enzymes (e.g., calpain) were activated by excessive $Ca^{2+}$, and the activated calpain then acted on downstream Bcl-2 family proteins to trigger the release of cytochrome-c from the mitochondria and activate the apoptotic pathway.[33] On the other hand, ER $Ca^{2+}$ release and cytosolic $Ca^{2+}$ elevation may cause mitochondrial $Ca^{2+}$ loading and result in mitochondria damage, which leads to cell death.[34,35] Our findings also suggest that the calcium concentration-dependent pathway may contribute to **b5**-induced caspase-3 activation and apoptosis in AW1 cells. As shown in Fig. 5, $Ca^{2+}$ can be increased by **b5** via the release of calcium ions from the internal stores. Flow cytometry analysis results showed that the ratio of apoptotic cells was clearly increased with **b5** treatment (Fig. 1). Furthermore, apoptosis-related proteins, including Bcl-2, Bax and Cyt-c, were detected (Fig. 3) and showed that the effects of **b5** treatment may occur primarily through mitochondrial pathways at early apoptotic stages, followed by a caspase-3-dependent processes (Fig. 4). Thus, these preliminary observations suggested that the elevation of $[Ca^{2+}]_i$ is involved in **b5**-induced apoptosis in AW1 cells.

## 3. Conclusion

Taken together, the results from this study show that pyrazole carboxamides with diarylamine-modified scaffold (**b5**) can inhibit cell proliferation and promote cell apoptosis by the mitochondrial pathway that is associated with elevation of intracellular calcium in a dose-dependent manner. The research provided useful information on **b5** as a potential apoptosis inducer, a finding that is essential for developing a novel series of compounds for use as insecticides. We realize that there are some problems that remain to be solved, most important of which is the interpretation of the mechanistic relationship between apoptosis induction and insecticidal activity. This feature will be our focus in future studies.

## 4. Experimental

### 4.1. Chemicals and reagents

Compounds **a1**–**a9**, **b1**–**b8**, and **c1**–**c10** were synthesized in our laboratory (Key laboratory of Bio-resources and eco-environment, Ministry of education, China).[5] Dimethyl sulfoxide (DMSO), phosphate buffer saline (PBS), thiazolyl blue tetrazolium bromide (MTT), Rhodamine 123 (Rh 123), Excell420 insect medium, Flou-3/AM dye, phenylmethanesulfonyl fluoride (PMSF), and RIPA lysis buffer were purchased from Sigma (St. Louis, MO, USA). Fetal bovine serum (FBS) was the product of Thermo Fisher Scientific Co., Ltd. (Utah, USA). The Caspase-3 assay kit and Annexin V-EGFP/PI apoptosis detection kit were purchased from Keygen Biotech Co., Ltd. (Nanjing, China). The antibodies we used were as follows

cytochrome-c, Bcl-2, Bax, β-actin and the secondary anti-rabbit antibody were purchased from Zen Bioscience Co., Ltd. (Chengdu,

China). All other reagents and chemicals used were of analytical grade and were purchased locally.

### 4.2. Cell line culture conditions

RP-HzVNC-AW1 cells were obtained from the Biological Control of Insects Research Laboratory at the United States Department of Agriculture. AW1 cells were derived from the ventral nerve cord of *Helicoverpa zea*.[7] The cell line was grown in Excell420 insect medium containing 10% fetal bovine serum, penicillin (50 U/ml) and streptomycin (50 μg/ml) (Gibco). AW1 cells were routinely maintained at 28 °C in 25 $cm^2$ culture flasks (Corning, Corning, NY, USA) and were sub-cultured every 5 days.[7]

### 4.3. Biological assay

Cytotoxic effects were assayed using 96-well flat-bottomed plastic microplates. Cells with a density of $1 \times 10^5$ cells per millilitre (100 μl) were seeded into each well. After overnight incubation, the cells were exposed to a series of pyrazole carboxamide compounds and fipronil (dissolved in DMSO) with different concentrations for 48 h. The cell viability was analysed by the MTT colorimetric assay method and calculated as the percent of absorbance in the control (0.1% DMSO treated cells).[36,37]

### 4.4. Apoptosis assay

Apoptosis-associated changes were examined by cytofluorometry (FACS Calibur, BD). The Annexin V-EGFP/PI apoptosis kit was used to assess apoptosis in the early and later events of cellular cytotoxicity. AW1 cells were seeded in six-well plates ($1 \times 10^5$ cells/ml) in 2 ml of Excell 420 insect medium. After 24 h treatment with 10, 50, or 100 μM of **b5** (0.1% DMSO used as control.), cells were harvested at 1000 rpm for 5 min and washed twice with PBS. The cells were then labelled with Annexin V-EGFP and PI for 15 min in the dark before being analysed by flowcytometry. Annexin V was set as the horizontal axis, and PI was set as the vertical axis. Mechanically damaged cells were in the upper left quadrant, apoptotic cells were in the upper right quadrant, normal cells were in the lower left quadrant and early apoptotic cells were in the lower right quadrant of the flow cytometric dot plot.

### 4.5. Mitochondrial membrane potential ($\Delta\Psi m$) analysis

The mitochondrial membrane potential was analysed using flow cytometry. After treatment with **b5** at concentrations of 10, 50, and 100 μM and 0.1% DMSO (the control) for 24 h, the AW1 cells were harvested and washed twice with PBS and then incubated with Rh 123 (2 μM for final concentration) in the dark for 60 min at 37 °C. The cells were collected by centrifugation after being washed twice with PBS to remove extracellular Rh 123, and the fluorescence intensity was measured by flow cytometry (Beckman Coulter Inc, USA) at an excitation wavelength of 507 nm and an emission wavelength of 529 nm.

### 4.6. Western blotting

We extracted the total protein from **b5**-treated cells (10, 50, and 100 μM for 24 h) using cold RIPA lysis buffer with 1 mM of PMSF, and the protein concentration was determined using the BCA protein assay method.[38] Then, 30 μg of protein samples was segregated by 15% SDS-PAGE and transferred to PVDF membranes using electrophoresis. The blocked blots in Tris-buffered saline-Tween with 5% non-fat dry milk at room temperature for 1 h were then treated for immunoblotting with primary antibodies for cytochrome c, Bcl-2, Bax, and β-actin and the HRP-conjugated secondary antibodies. The immune-reactive proteins were visualized using the Super Signal West Pico Trial Kit (Corning).

### 4.7. Measurement of caspase-3 activity

The activity of caspase-3 was evaluated on the basis of spectrophotometric detection with the Caspase-3 assay kit. After treatment with compound **b5** at 10, 50, and 100 μM (0.1% DMSO used as control) for 0, 12, and 24 h, the cells were collected by centrifugation at 10000 rpm for 10 min at 4 °C and were subsequently resuspended in cell lysis buffer and incubated on ice for 1 h. The resulting supernatant (50 μl) was mixed in 2 × Reaction Buffer (50 μl) and Caspase-3 Substrate (5 μl) and was incubated at 37 °C for 4 h. Finally, caspase-3 activity was measured at 405 nm with a microplate reader (BioTek, USA).

### 4.8. Measurement of intracellular calcium concentration

The cells were collected by centrifugation after being washed once with D-Hanks [(mg/L)

NaCl 800, KCl 400, $KH_2PO_4$ 60, $NaHCO_3$ 350, $Na_2HPO_4{\cdot}12H_2O$ 132, and D-glucose 1000, buffered to pH 7.0] to remove the extracellular culture medium. The cells were subsequently resuspended with dye loading buffer containing 4 μM of fluo-3/AM and 0.04% pluronic F-127 in D-Hanks.[39] After a 45-min incubation in dye loading buffer, cells were washed twice with D-Hanks buffer. The 96-well clear-bottomed black plates (Corning) were then transferred to a fluorescence plate reader (Thermo Scientific). Cells were excited at 488 nm, and the 530 nm fluorescence emission was recorded. Fluorescence readings were taken once every 30 s for 15 min after the **b5** compound solution was added to each well yielding a final concentration of 10, 50, and 100 μM. Fluorescence values were expressed as F/F0, with F0 being the resting (or baseline) fluorescence and F being the change in fluorescence from baseline after the application of the drug.

### 4.9. Data analysis

Each experiment was repeated at least three times. The data were analysed with SPSS V17 and Excel 2013. Statistical significance was determined by ANOVA and Student's *t*-test ($^{*}P \leq 0.05$, $^{**}P \leq 0.01$). The results were expressed as the mean ± SD. The error bars represent SEM.

## Declarations of interest

None.

## Acknowledgments

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## A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.bmc.2018.03.010.

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