HETEROCYCLES, Vol. 90, No. 2, 2015, pp. 1317 - 1322. © 2015 The Japan Institute of Heterocyclic Chemistry
Received, 30th June, 2014, Accepted, 30th July, 2014, Published online, 4th August, 2014
DOI: 10.3987/COM-14-S(K)68

ISOLATION OF ALKAMIDES WITH DEATH RECEPTOR−ENHANCING ACTIVITIES FROM *PIPER CHABA*

Hoque Tahmina,[1] Kazufumi Toume,[1] Midori A. Arai,[1] Samir K. Sadhu,[2] Firoj Ahmed,[3] and Masami Ishibashi[1*]

[1]Graduate School of Pharmaceutical Sciences, Chiba University, 1-8-1 Inohana, Chuo-ku, Chiba 260-8675, Japan, [2]Pharmacy Discipline, Life Science School, Khulna University, Khulna 9208, Bangladesh, [3]Department of Pharmaceutical Chemistry, University of Dhaka, Dhaka 1000, Bangladesh

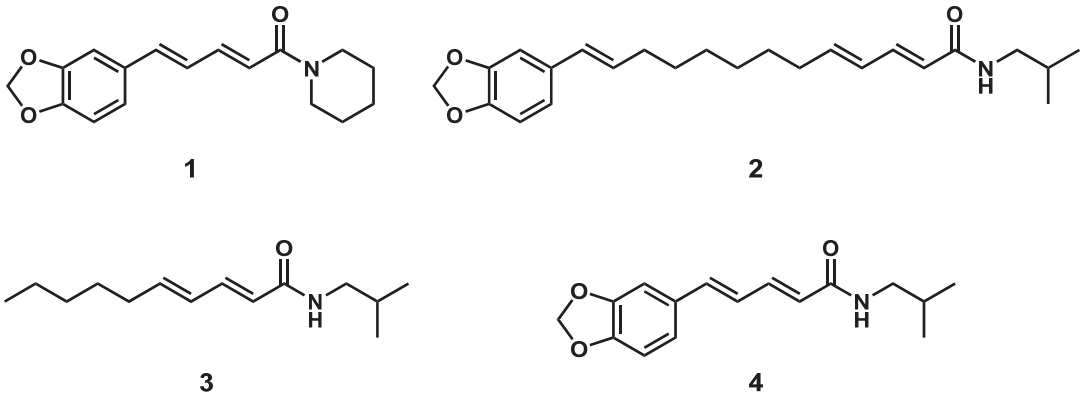
Abstract – In the screening program for natural bioactive compounds that enhance the expression of death receptor 5 (DR5), activity-guided fractionation of the *Piper chaba* (Piperaceae) root MeOH extract led to the isolation of four alkamides (**1**-**4**). Compounds **1**, **3**, and **4** enhanced DR5 promoter activity while pellitorine (**3**) exhibited TRAIL-resistance-overcoming activity.

Tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL), a member of the TNF superfamily, has emerged as a promising anti-cancer agent that is capable of killing tumor cells selectively without significantly affecting normal cells.[1] TRAIL can induce apoptosis by binding to death receptors (DR) to form a death-inducing signaling complex (DISC), and this is followed by the activation of caspase-8 and caspase-3.[1] Nevertheless, a large number of cancer cells, especially serious malignant tumors, are resistant to TRAIL. Therefore, overcoming TRAIL resistance and elucidating the mechanisms involved in TRAIL resistance are important in the discovery of anti-cancer drugs. A previous study reported that a decrease in cell surface DR4 and DR5 levels, increase in decoy receptors, and enhancement in apoptosis inhibitors, including cellular FLICE-like inhibitory protein (cFLIP) and B-cell CLL/lymphoma 2 (BCL-2), are the mechanisms responsible for TRAIL resistance.[1] Therefore, identifying the small molecules that enhance DR5 may represent one solution to overcome TRAIL resistance.

We examined the MeOH extracts of different kinds of plant resources collected from Bangladesh and Thailand in our screening program for bioactive natural compounds that induce tumor-selective apoptosis[2] and identified several natural small molecules as enhancers of death receptor 5 (DR5) promoter activity, including cycloartane triterpenoids,[3,4] ingol diterpenoids,[4] isoflavones,[5] flavonoids,[3] and chalcones.[6] In our continuous screening, the MeOH extract of the *Piper chaba* (Piperaceae) root was

shown to enhance DR5 promoter activity. *P. chaba* is a climbling, glabrous shrub that is found in various areas of India and the Malaya Islands, and has many folklore uses, including as an alexiteric and for asthma, bronchitis, and lung tuberculosis.[7] Several alkamides including piperine, silvatine, piplartine, and piperlonguminine have been isolated from *P. chaba*.[8] We here described the activity-guided isolation of the MeOH extract of the *Piper chaba* root, and enhancements in the DR5 promoter and TRAIL-resistance-overcoming activities of these isolates.

In the screening assay, DR5 promoter activity were evaluated in DLD-1/SacI cells using the luciferase assay.[14] The MeOH extract of the *Piper chaba* root (50 μg/mL) had 3.3-fold higher DR5 promoter activity. Activity-guided isolation of this extract by silica gel and ODS column chromatography yielded four alkamides: piperine[9,10] (**1**), guineensine[9,11] (**2**), pellitorine[9,12] (**3**), and piperlonguminine[9,13] (**4**). These compounds were identified by comparing their spectroscopic data with values reported in the literature.



The effects of the identified compounds on DR5 promoter activity were evaluated. In this assay, luteolin,[15,16] which was previously shown to enhance the expression of DR5, was used as a positive control. As shown in Figure 1, **1**, **3**, and **4** enhanced DR5 promoter activity. **3** exhibited potent activity that was 2.7-fold higher at 50 μM, whereas **1** enhanced by 2.5-fold at 30 μM and **4** enhanced by 2.1-fold at 50 μM, respectively.

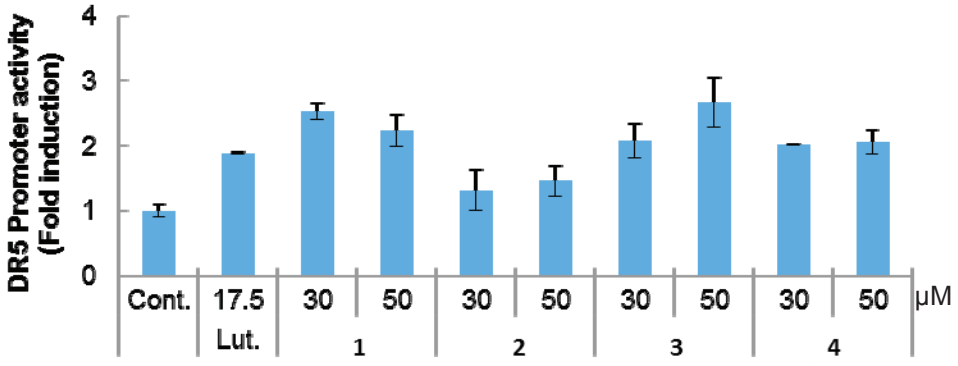


Figure 1. Effects of **1**-**4** on DR5 promoter activity

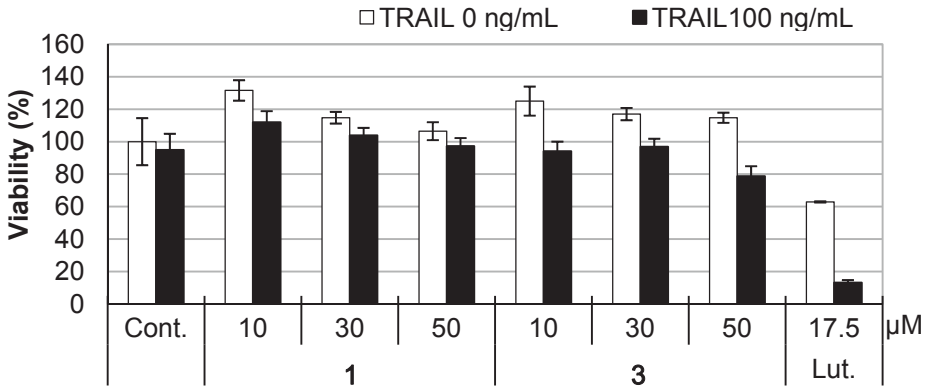


Figure 2. TRAIL-resistance-overcoming activities of **1** and **3**

In the TRAIL signaling pathway, TRAIL binds to its receptors, DR4 and DR5 to form the death-inducing signaling complex (DISC) and subsequently activates caspases, leading to apoptotic cell death. Therefore, we assessed the TRAIL-resistance-overcoming activities of **1** and **3**. The AGS cell line, TRAIL-resistant gastric adenocarcinoma cells, was used in this assay. As shown in Figure 2, AGS cells treated with TRAIL (100 ng/mL) for 24 h had slightly reduced in cell viability (5%). Luteolin[16] at 17.5 μM, which was used as a positive control, led to a 50% reduction in cell viability when administrated in combination with TRAIL over the treatment with luteolin alone. The combined treatment with **3** (50 μM) and TRAIL (100 ng/mL) led to a decrease in cell viability over that with the compound only (without TRAIL), showing activity to overcome TRAIL resistance. On the other hand, the combined treatment with **1** and TRAIL did not significantly decrease cell viability (only a 20% decrease) over that with the compound alone. These results indicated that enhanced DR5 promoter activity contributed to the TRAIL-resistance-overcoming activity of **3**. While **1** enhanced DR5 promoter activity, the TRAIL-resistance-overcoming activity of **1** was not detected, indicating that **1** may affect other signal molecules involved in cell survival. The enhancing effects of piperine (**1**) on the efficacy of TRAIL-based therapies in triple-negative breast cancer[17] and vascular barrier protective effects of piperlonguminine (**4**) have recently been reported.[18] However, the effects of these compounds (**1**-**4**) on the expression of DR5 or TRAIL signaling have not yet been reported. To the best of our knowledge, this is the first study on alkamides to show that the DR5 promoter and TRAIL-resistance-overcoming activities of **3** were enhanced, thereby implying the therapeutic potential of **3** in combined use with TRAIL against TRAIL-resistant gastric cancer.

EXPERIMENTAL

General Experimental Procedure. Column chromatography was performed using silica gel 60N (Kanto Chemical Co., Inc.) and Chromatorex ODS (Fuji Silysia Chemical Ltd., Kasugai, Japan).

Plant Materials. The roots of *P. chaba* were collected from Bangladesh. A voucher specimen (KKB109)

was deposited in the Department of Natural Products Chemistry, Graduate School of Pharmaceutical Sciences, Chiba University.

Cell cultures

DLD-1/*Sac*I cells were a generous gift from Prof. Toshiyuki Sakai (Kyoto Prefectural University of Medicine). AGS cells were purchased from ATCC. Cells were cultured in Roswell Park Memorial Institute (RPMI-1640) medium (Wako) with 10% fetal bovine serum (FBS) in a humidified incubator at 37 °C in 5% CO_2/95% air. The purity of all compounds tested was greater than 95%, as determined by TLC and NMR. Luteolin (purity ≥ 98%, Sigma Aldrich) was used as a positive control.

DR5 promoter activity assay

The assay procedure was as described previously.[14] Briefly, DLD-1/*Sac*I cells (2 x 10^5 per well), a human colon cancer cell line that had been stably transfected with the DR5 promoter-luciferase reporter plasmid pDR5/*Sac*I,[19] were treated with different concentrations of each compound for 24 h at 37 °C. After the removal of medium containing the compound, the cells were lysed in Cell Culture Lysis Reagent (Promega). The chemiluminescence of the lysate was then measured for 10 s as relative light units using a Luminoskan Ascent (Thermo Scientific), and DR5 promoter activity was evaluated by comparing the chemiluminescence of the sample with that of the control (cells treated with DMSO).

Isolation of the constituents. The dried root of *P. chaba* (126 g) was extracted by MeOH to afford the MeOH crude extract (3.5 g). The MeOH extract was subjected to silica gel column chromatography (φ20 × 400 mm), eluting with the hexane:EtOAc gradient system (9:1 to 0:1, acetone, and MeOH) to give fractions 1A-1M. Fraction 1D (42.6 mg), 1F (18.3 mg), and 1H (509 mg) eluted with hexane:EtOAc (7:3) were identified as pellitorine (**3**), guineensine (**2**), and piperine (**1**), respectively. An active fraction 1I (62.1 mg), which was eluted with hexane:EtOAc (6:4-5:5) and showed a 3.5-fold increase in DR5 promoter activity at 10 μg/mL, was subjected to ODS column chromatography using the MeOH:H_2O (8:2-1:0) system to yield 2A-2E. Fraction 2A (40.3 mg), which was eluted with MeOH:H_2O (8:2), was again subjected to ODS column chromatography using the MeOH:H_2O (65:35-1:0) system to yield 3A-3E. Fraction 3B (7.1 mg), which was eluted with MeOH:H_2O (68:32-7:3), was identified as piperlonguminine (**4**).

TRAIL-resistance-overcoming activity assay

AGS cells were seeded in a 96-well culture plate (6 × 10^3 cells per well) in 200 μL of RPMI-1620 medium containing FBS and then incubated for 24 h. Test samples at different doses with or without TRAIL (100 ng/mL) were added to each well and incubated for 24 h. They were then treated with fluorescein diacetate (Wako, Osaka, Japan) in PBS buffer (10 μg/mL), and fluorescence was detected after a 1-h incubation using Fluoroskan Ascent (Thermo). Assays were performed at least in triplicate. Data are shown as the mean ± SD.

ACKNOWLEDGEMENTS

This study was supported by Grants-in-Aid for Scientific Research from the Japan Society for the Promotion of Science (JSPS), a Grant-in-Aid for Scientific Research on Innovative Areas "Chemical Biology of Natural Products" from The Ministry of Education, Culture, Sports, Science and Technology (MEXT), Japan, the Cosmetology Research Foundation, the Hamaguchi Foundation for the Advancement of Biochemistry and the Uehara Memorial Foundation.

REFERENCES

1. G. Mellier, S. Huang, K. Shenoy, and S. Pervaiz, *Mol. Aspects Med.*, 2010, **31**, 93.
2. M. Ishibashi and M. A. Arai, *J. Synth. Org. Chem. Jpn.*, 2009, **67**, 1094; M. Ishibashi and T. Ohtsuki, *Med. Res. Rev.*, 2008, **28**, 688.
3. K. Toume, T. Nakazawa, T. Ohtsuki, M. A. Arai, T. Koyano, T. Kowithayakorn, and M. Ishibashi, *J. Nat. Prod.*, 2011, **74**, 249.
4. K. Toume, T. Nakazawa, T. Hoque, T. Ohtsuki, M. A. Arai, T. Koyano, T. Kowithayakorn, and M. Ishibashi, *Planta Med.*, 2012, **78**, 1370.
5. H. Kikuchi, T. Ohtsuki, T. Koyano, T. Kowithayakorn, T. Sakai, and M. Ishibashi, *Bioorg. Med. Chem.*, 2009, **17**, 1181.
6. T. Ohtsuki, H. Kikuchi, T. Koyano, T. Kowithayakorn, T. Sakai, and M. Ishibashi, *Bioorg. Med. Chem.*, 2009, **17**, 6748.
7. M. Taufiq-Ur-Rahman, J. A. Shilpi, M. Ahmed, and C. Faiz Hossain, *J. Ethnopharmacol.*, 2005, **99**, 203.
8. A. Patra and A. Ghosh, *Phytochemistry*, 1974, **13**, 2889.
9. S. Wu, C. Sun, S. Pei, Y. Lu, and Y. Pan, *J. Chromatogr. A*, 2004, **1040**, 193.
10. H. C. Oersted, *Journal für Chemie und Physik*, 1820, **29**, 80.
11. J. I. Okogun and D. E. U. Ekong, *J. Chem. Soc., Perkin Trans. 1*, 1974, 2195.
12. M. Jacobson, *J. Am. Chem. Soc.*, 1949, **71**, 366.
13. M. E. Synerholm, A. Hartzell, and J. M. Arthur, *Contrib. Boyce Thompson Inst.*, 1945, **13**, 433.
14. H. Kikuchi, T. Ohtsuki, T. Koyano, T. Kowithayakorn, T. Sakai, and M. Ishibashi, *J. Nat. Prod.*, 2007, **70**, 1910.
15. M. Horinaka, T. Yoshida, T. Shiraishi, S. Nakata, M. Wakada, R. Nakanishi, H. Nishino, H. Matsui, and T. Sakai, *Oncogene*, 2005, **24**, 7180.
16. M. Horinaka, T. Yoshida, T. Shiraishi, S. Nakata, M. Wakada, R. Nakanishi, H. Nishino, and T. Sakai, *Biochem. Biophys. Res. Commun.*, 2005, **333**, 833.
17. S. Abdelhamed, S. Yokoyama, A. Refaat, K. Ogura, H. Yagita, S. Awale, and I. Saiki, *Anticancer*

Res., 2014, **34**, 1893.

18. S. K. Ku, J. A. Kim, and J. S. Bae, *Inflamm. Res.*, 2014, **63**, 369.
19. T. Yoshida, A. Maeda, N. Tani, and T. Sakai, *FEBS Lett.*, 2001, **507**, 381.