Phytochemistry Letters 11 (2015) 280–285

Contents lists available at ScienceDirect

Phytochemistry Letters

journal homepage: www.elsevier.com/locate/phytol

Limonoids with Wnt signal inhibitory activity isolated from the fruits of *Azadirachta excelsa*

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ARTICLE INFO

Article history:
Received 19 November 2014
Received in revised form 15 January 2015
Accepted 20 January 2015
Available online 31 January 2015

Keywords:
β-Catenin
Limonoids
Adenomatous polyposis coli
Colorectal cancer

ABSTRACT

The Wnt signal regulates various biological processes, and its aberrant activation is associated with the development of diseases. Thus, inhibiting the Wnt signal provides a promising strategy to treat these diseases. Our cell-based luciferase assay system, which targets the Wnt signal (TOP assay), revealed that *Azadirachta excelsa* inhibited the Wnt signal. The activity-guided isolation of the MeOH fruit extract of *A. excelsa* provided one new (**1**) and seven known (**2**–**8**) limonoids. Their structures were elucidated based on their spectroscopic data, and their NMR data were compared with those in the literature. Compounds **3**–**6** potently inhibited the Wnt signal with IC_{50} values of 127 nM, 300 nM, 252 nM, and 121 nM, respectively. Compound **4** exhibited selective cytotoxicity against AGS and HCT116. Western blot analysis showed that **4** did not affect the level or localization of β-catenin, but downregulated the level of c-myc. Our results suggested that **4** may have inhibited the Wnt signal by affecting the components downstream of β-catenin.

1. Introduction

The Wnt signaling pathway is involved in various embryonic developmental and cellular processes (Clevers and Nusse, 2012). In the absence of the Wnt protein, β-catenin levels in normal cells are regulated by the action of a destruction complex consisting of glycogen synthase kinase 3β (GSK3β), casein kinase 1α (CK1α), and adenomatous polyposis coli (APC). GSK3β and CK1α phosphorylate β-catenin (Liu et al., 2002) which is then degraded via the ubiquitin-proteasome pathway (Aberle et al., 1997). However, inactivation of this destruction complex due to either stimulation of the Wnt protein or a mutation in the components of the destruction complex has been shown to result in the stabilization and accumulation of β-catenin (Li et al., 2012). Accumulated β-catenin enters the nucleus and forms a complex with TCF and other co-activators, such as Bcl9, pygopus, and CREBB-binding protein (CBP), to activate the transcription of important proliferation genes (Clevers and Nusse, 2012). However, the deregulated activation of the Wnt signal has been associated with several human diseases such as cancer (Clevers and Nusse, 2012). Most colorectal cancer cells have truncated adenomatous polyposis coli (APC) or mutated β-catenin, which stabilizes β-catenin and activates the Wnt signal (Morin et al., 1997). Thus, inhibiting the accumulation of β-catenin accumulation or formation of the TCF/β-catenin complex could represent a good strategy for preventing these diseases.

Several compounds had been shown to inhibit the Wnt signal, some of which were derived from natural resources. The flavonoid quercetin (Park et al., 2005), phenolic compound curcumin (Ryu et al., 2008), and carbazole alkaloid murrayafoline A (Choi et al., 2010) are some of the plant-derived compounds that inhibit the Wnt signal. Our group also had previously described a naturally derived compound that inhibited the Wnt signal and also exhibited cytotoxicity against Wnt-dependent cancer cells (Park et al., 2014; Toume et al., 2013). In our continuous work to search for Wnt signal inhibitors from natural resources, we identified *Azadirachta excelsa* as a potential source from our screening study. *A. excelsa* belongs to the family Meliaceae, which is known to be a source of limonoid-type compounds. Meliaceous limonoids have been shown to possess antineoplastic, antimicrobial, and antiprotozoal

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http://dx.doi.org/10.1016/j.phytol.2015.01.015
1874-3900/

properties (Tan and Luo, 2011). In the present study, our activity-guided isolation of the *A. excelsa* fruit extract led to the isolation of one new (**1**) and seven known (**2**–**8**) limonoids that potently inhibited the Wnt signal.

2. Results and discussion

Wnt/β-catenin signal inhibitory activity was evaluated using a cell-based luciferase assay (TOP assay). The reporter assay cell (STF/293) was an HEK293 cell line stably transfected with SuperTOPflash, a luciferase plasmid containing seven copies of the TCF binding sites (TOP). Lithium chloride was added during the treatment to activate the Wnt signal in STF/293 cells by inhibiting GSK3β (Stambolic et al., 1996). β-Catenin accumulates and translocates to the nucleus. β-Catenin then forms a complex with TCF, and initiates the transcription of luciferase. Using this assay system, our screening study revealed that the fruit extract of *A. excelsa* inhibited the Wnt signal by 79% at 10 μg/mL. The viability of the reporter cells was 97% at the same concentration, which indicated that the observed decrease in transcriptional activity was not due to decrease in the number of reporter cells. Activity-guided fractionation using chromatographic techniques of the MeOH extract of *A. excelsa* (fruits) led to the isolation of one new (**1**) and seven known (**2**–**8**) trichillin-type limonoids. The new compound **1** was named as mahaneemin after the local name of this plant in Bangladesh "mahaneem".

Mahaneemin (**1**) was isolated as a colorless amorphous solid. It had the molecular formula $C_{34}H_{44}O_{12}$ based on HRESIMS data. It gave an intense $[M+Na]^+$ peak at *m/z* 667.2742, corresponding to $C_{34}H_{44}O_{12}Na$ (Δ + 1.2 mmu). Based on its IR spectra, absorption peaks at 3489 and 1743 cm^{-1} indicated the presence of hydroxyl and carbonyl groups, respectively. Its ^1H and ^{13}C NMR spectra had signals for a typical tetranortriterpenoid (Table 1). The presence of the peaks δ_H 7.17 (s), 7.36 (t), and 6.17 (br s) suggested the presence of a β-furan-ring. The presence of two acetate groups was indicated by signals at δ_H 1.99 (3H, s) and 2.08 (3H, s). A comparison of the NMR spectra of **1** and the reported compound meliatoosenin I (**2**) (Zhang et al., 2012) showed that they had similar skeletons, except for the ester moiety at C-29. C-29 in **2** was attached to 2-methylbutanoyl, whereas **1** had a methylpropionyl group at this position. The attachment of the methylpropionyl group to C-29 was indicated by the HMBC correlation between H-29 and C-1′. The configuration at C-29 of **1** was determined as the same as that of **2**, based from the chemical shift at H-3. The chemical shift of H-3 is δ 5.52 which would suggest that the methylpropanoyl group is at the *exo*-position. In general for trichillin-type limonoids, 3-H signal appears more upfield ($\sim\delta$ 4.8) in the *endo*-configuration as compared to the *exo*-isomer ($\sim\delta$ 5.3) (Huang et al., 1994).

The other isolated compounds were identified as meliatoosenin I (**2**) (Zhang et al., 2012), 29-deacetylsendanin (**3**, C-29 epimeric mixture, 1:1) (Itokawa et al., 1995), trichillin H (**4**) (Nakatani et al., 1994), 12-*O*-acetylazedarachin B (**5**) (Huang et al., 1994), meliatoxin A_1 (**6**) (Macleod et al., 1990; Oelrichs et al., 1983), meliatoxin B_2 (**7**) (Macleod et al., 1990; Oelrichs et al., 1983) and meliatoxin B_1 (**8**) (Macleod et al., 1990; Oelrichs et al., 1983).

The eight isolated compounds inhibited the TOP activity in a concentration-dependent manner without affecting the activity of the control reporter (FOP). The FOP plasmid contained a mutant TCF binding site, and did not respond to the active Wnt signal. These results suggested that the compounds specifically inhibited TOP activity, and, thus, exhibited TCF/β-catenin transcription inhibitory activity. Compounds **3** and **6** showed the strongest inhibitory activity with IC_{50} values of 127 and 121 nM, respectively (Fig. 1 and Table 2). Compounds **5** (IC_{50} 252 nM) and **4** (300 nM) also showed relatively strong inhibitory activity. Furthermore, the 14,15-epoxide moiety may be an important functional group for the inhibitory activities of compounds **3**–**6**. When C-15 was replaced with a hydroxyl (**1**, **2**) or ketone (**7**, **8**) group, inhibitory activity was reduced.

Table 1
NMR spectroscopic data for compound **1** (in $CDCl_3$, 600 MHz).

Position	**1**	
	δ_H (*J* in Hz)	δ_C (*J* in Hz)
1	4.72 (1H, brs)	70.7
2	5.87 (1H, t, 4.8)	68.8
3	5.52 (1H, d, 4.8)	72.7
4		41.5
5	2.73 (1H, dd, 13.2, 5.4)	31.5
6	1.78 (1H, m)	23.1
	1.92 (1H, m)	
7	4.95 (1H, d, 3)	81.2
8		45.2
9	4.48 (1H, s)	52.1
10		39.8
11		208.5
12a	2.31 (1H, d, 19)	52.0
12b	2.48 (1H, d, 19)	
13		46.2
14		97.5
15	4.89 (1H, s)	77.0
16	1.97 (1H, m)	37.6
	1.87 (1H, m)	
17	3.16 (1H, dd, 11.4, 5.4)	45.0
18	1.03 (3H, s)	21.6
19	4.05, 4.02 (2H, ABq, 12)	64.2
20		124.1
21	7.17 (1H, s)	139.5
22	6.17 (1H, brs)	110.9
23	7.36 (1H, t, 1.8)	143.1
28	0.77 (3H, s)	17.1
29	5.69 (3H, s)	92.7
30	1.23 (3H, s)	17.0
2-$\underline{CH_3}CO$	1.99 (3H, s)	20.7
2-$CH_3\underline{CO}$		168.9
3-$\underline{CH_3}CO$	2.08 (3H, s)	20.9
3-$CH_3\underline{CO}$		170.1
1′		175.7
2′	2.59 (1H, quin, 7)	34.1
3′	1.20 (3H, d, 7)	18.7
4′	1.18 (3H, d, 7)	18.6
1-OH	2.29 (1H, brs)	

We then investigated whether compounds **3**–**5** could affect the viability of the gastrointestinal cancer cells SW480 (colon), HCT116 (colon), DLD1 (colon), and AGS (gastric), all of which possess an active Wnt signal (Ikenoue et al., 2002; Morin et al., 1997). We also determined their cytotoxic activity in Wnt-independent colon cancer RKO cells and the non-cancer cell line HEK293. After a 48-h treatment, compound **3** exhibited the strongest cytotoxicity against HCT116 among the compounds tested with an IC_{50} value of 24.9 nM (Fig. 2 and Table 3). Compounds **3** and **5** were also cytotoxic against the Wnt-independent RKO cancer cell with IC_{50} values of 192 nM and 331 nM, respectively. On the other hand, compound **4** exhibited cytotoxicity against Wnt-dependent AGS (IC_{50} 239 nM) and HCT116 (IC_{50} 161 nM). Although its IC_{50} values for these two cancer cells were higher than the observed IC_{50} values of either compounds **3** or **5**, its IC_{50} values for the Wnt-independent RKO as well as the non-cancer cell line were >400 nM (Table 3). Compounds **3**–**5** did not markedly decrease the viability of SW480 and DLD1 cells (IC_{50} >400 nM). This result indicated that, among the compounds tested, **4** was selectively cytotoxic against Wnt-dependent cancer cells, particularly AGS and HCT116.

Since **4** was selectively cytotoxic against Wnt-dependent AGS and HCT116, we investigated its effects on β-catenin using Western blotting analysis. Western blot analysis revealed that **4**