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Generation of 'Unnatural Natural Product' library and identification of a small molecule inhibitor of XIAP

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ABSTRACT

Natural products have been utilized for drug discovery. To increase the source diversity, we generated a new chemical library consisting of chemically modified microbial metabolites termed 'Unnatural Natural Products' by chemical conversion of microbial metabolites in crude broth extracts followed by purification of reaction products with the LC-photo diode array–MS system. Using this library, we discovered an XIAP inhibitor, C38OX6, which restored XIAP-suppressed enzymatic activity of caspase-3 in vitro. Furthermore, C38OX6 sensitized cancer cells to anticancer drugs, whereas the unconverted natural product did not. These findings suggest that our library could be a useful source for drug seeds.

1. Introduction

It is now recognized that evasion of apoptosis is a hallmark of human cancer. One cause of resistance to apoptosis is overexpression of anti-apoptotic proteins, including inhibitor of apoptosis (IAP) family proteins. X-linked inhibitor of apoptosis (XIAP) is a member of IAP family proteins, and overexpression of XIAP is often reported in several types of human cancer, such as acute myeloid leukemia (AML) and pancreatic cancer.[1] XIAP is an endogenous direct inhibitor of caspase-3, -7, and -9, which are key apoptosis signal mediators. In the structure of XIAP, three baculovirus IAP repeat (BIR) domains are conserved in IAP family proteins. The BIR2 domain with its flanking region of XIAP binds and inhibits active caspase-3/-7, whereas the BIR3 domain binds and inhibits caspase-9.[2–4] By inhibiting the enzymatic activities of these effector and initiator caspases, XIAP maintains cancer cell survival and renders cells resistant to apoptosis induction by radiation and anticancer drugs. Although XIAP is expressed in some normal tissues, no overt phenotype was detected in XIAP-deficient mice,[5] indicating that XIAP would be a promising molecular target for cancer therapies; therefore, we searched for compounds that inhibited XIAP function.

Traditionally, natural products have been a major source of lead molecules in drug development because natural products are highly diverse and often provide highly specific biological activities; however, obtaining drug-lead molecules from natural sources requires us to complete a series of time-consuming steps, including screening of extract libraries, bioassay-guided isolation, structure elucidation, and subsequent production scale-up. Therefore, over the past decade, combinatorial chemistry has become the major source of lead molecules in drug discovery; however, despite the increased speed of synthesis, this changeover from traditional to combinatorial synthesis has not yielded any real increase in the number of lead optimization candidates or drugs.[6] This apparent lack of productivity may be explained by the structural differences between natural and combinatorial compounds in that combinatorial compounds are substantially less diverse than natural products from many viewpoints, such as the number of chiral centers, the prevalence of aromatic rings, the introduction of complex ring systems, and the degree of saturation of the molecule as well as the number and ratios of different heteroatoms. Indeed, in the chemical space, natural products occupied a wide region, and the distribution of natural products (but not combinatorial compounds) is quite similar to that of approved drugs.[7] Therefore, natural

Abbreviations: UNP, Unnatural Natural Product; PDA, photo diode array.

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products have been, yet again, recognized to play a dominant role in the discovery of leads for the development of drugs for the treatment of human diseases, and several industrial and academic groups are using natural products to construct high-quality screening libraries.[8,9] Methods have been developed to generate large and diverse natural product libraries optimized for high-throughput screening and for a fast discovery process. Recently, we have also constructed a semi-natural product library whose components are chemically modified microbial metabolites which are referred to as 'Unnatural Natural Products (UNPs)'. Here we report the details of our new method to generate the UNP library and discovery of a new XIAP inhibitor.

2. Results

2.1. Generation of 'Unnatural Natural Product' library

To generate the 'Unnatural Natural Product (UNP)' library, we devised a series of unique procedures consisting of (i) preparation of actinomycetal fermentation broth extracts, (ii) chemical conversion of actinomycetal metabolites in crude broth extracts, (iii) detection of newly generated components which are referred to as UNPs, and (iv) isolation of UNPs by chromatography (Fig. 1A). In these procedures, chemical conversion of actinomycetal metabolites was the key step; therefore, we first examined the reaction

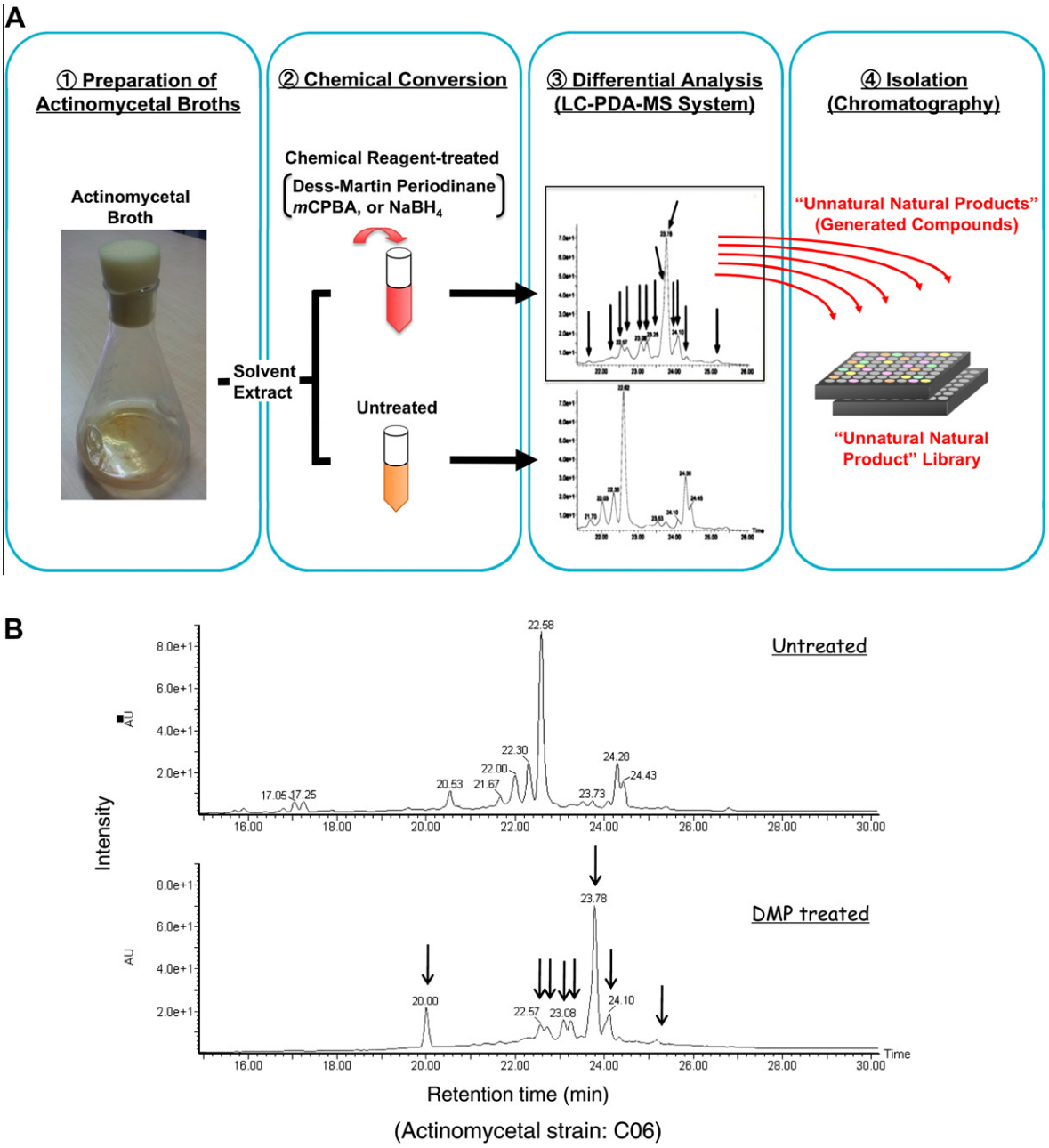

Figure 1. Generation of 'Unnatural Natural Product (UNP)' library. (A) Strategy for generation of UNP library. (B-D) Representative photo diode array (PDA) chromatogram of DMP, *m*CPBA or $NaBH_4$-treated or untreated actinomycetal broth extracts. Each actinomycetal broth extract was stirred with or without DMP in CH_2Cl_2 for 3 h (B), *m*CPBA in CH_2Cl_2 for 3 h (C), or $NaBH_4$ in MeOH for 1 h (D) at room temperature. Subsequently, the extracts of the reaction mixtures were analyzed by the LC–PDA–MS system (column: ODS, LC: 30–100% MeOH/H_2O (linear gradient) during 0–15 min and 100% MeOH during 15–45 min, detection: 220–600 nm). The peaks of UNPs were distinguished from those of unconverted natural products on the basis of their unique UV and MS spectra and retention time on HPLC. Representative peaks of UNPs are indicated with arrows.

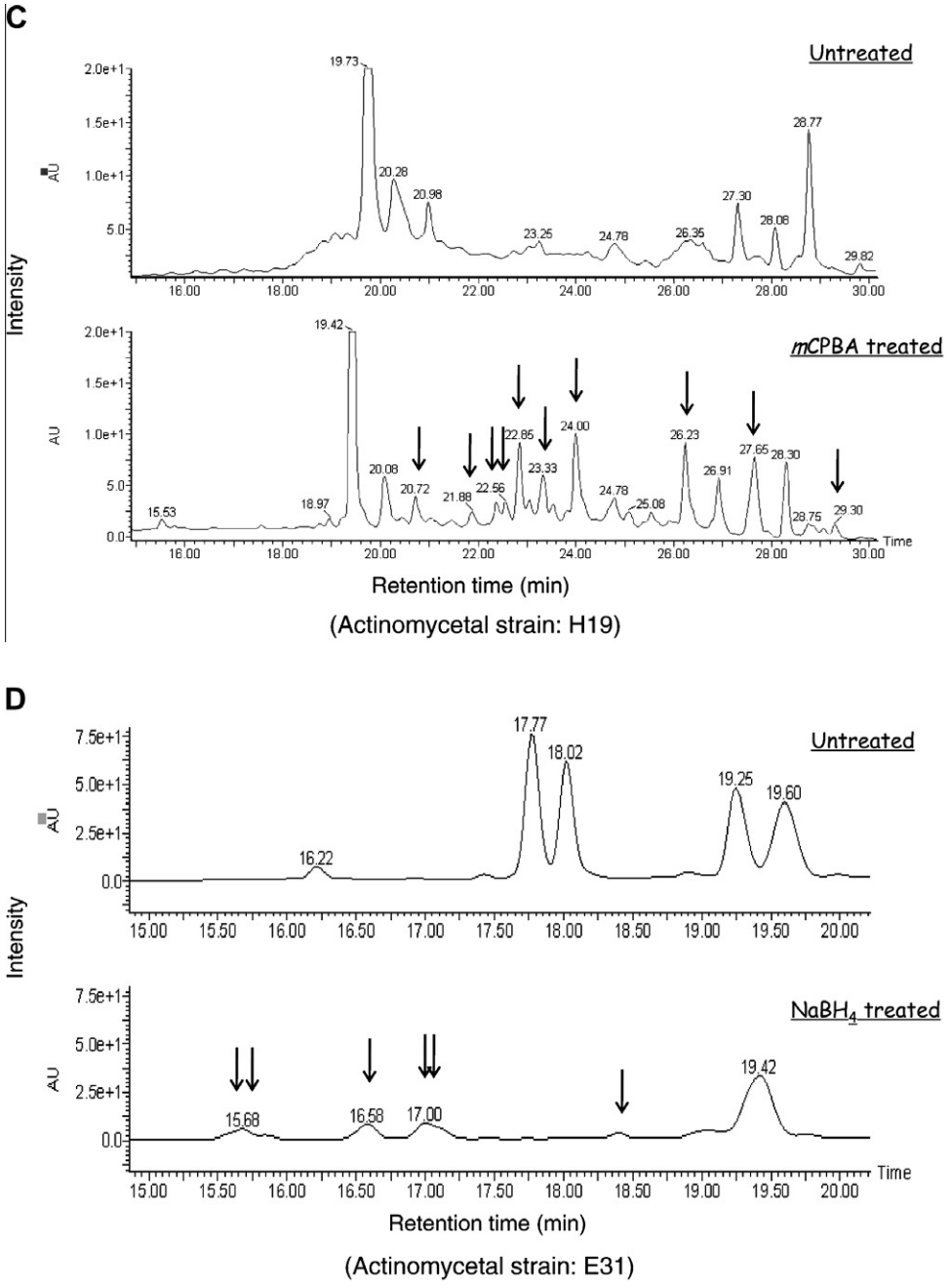

Fig. 1 (*continued*)

conditions, which efficiently converted diverse actinomycetal metabolites in the broth extracts without disruption of their natural product skeletons. As some natural products are unstable at extreme temperature and pH, we selected three types of relatively mild chemical reactions, oxidation by Dess–Martin periodinane (DMP), epoxidation by *meta*-chloroperbenzoic acid (*m*CPBA), and reduction by $NaBH_4$. Reaction products were separated through the HPLC unit, followed by spectral analyses in the detector units which gave us UV/vis and ESI-MS spectra (liquid chromatography-photo diode array–mass spectrometry (LC–PDA–MS) system), and distinguished from the unconverted actinomycetal metabolites (natural products). Each actinomycetal strain produces its own secondary metabolites with a huge variety of structures; therefore, by the addition of an equal weight of DMP or *m*CPBA to the actinomycetal broth extracts, many UNPs were produced through 3 h-chemical reaction at room temperature in many cases; however, the addition of excessive amounts of the chemical reagents caused the decomposition of UNPs. In a reduction reaction, chemical conversion was successful for 1 h by the addition of 10 times the amount of $NaBH_4$. Representative PDA chromatograms of the broth extracts with or without addition of chemical reagents are shown in Figure 1B–D. For the construction of the UNP library, UNPs in the reaction mixture were roughly purified by column chromatography on silica gel, and were further purified through the LC–PDA–MS system. The purity of isolated UNPs was also checked by the LC–PDA–MS system and the physico-chemical properties such as the retention times on the HPLC, UV/vis spectra, and ESI-MS spectra were stored to construct a database. Although the structure of each UNP in the library has not been elucidated, physico-chemical properties provide valuable structural information on each UNP.

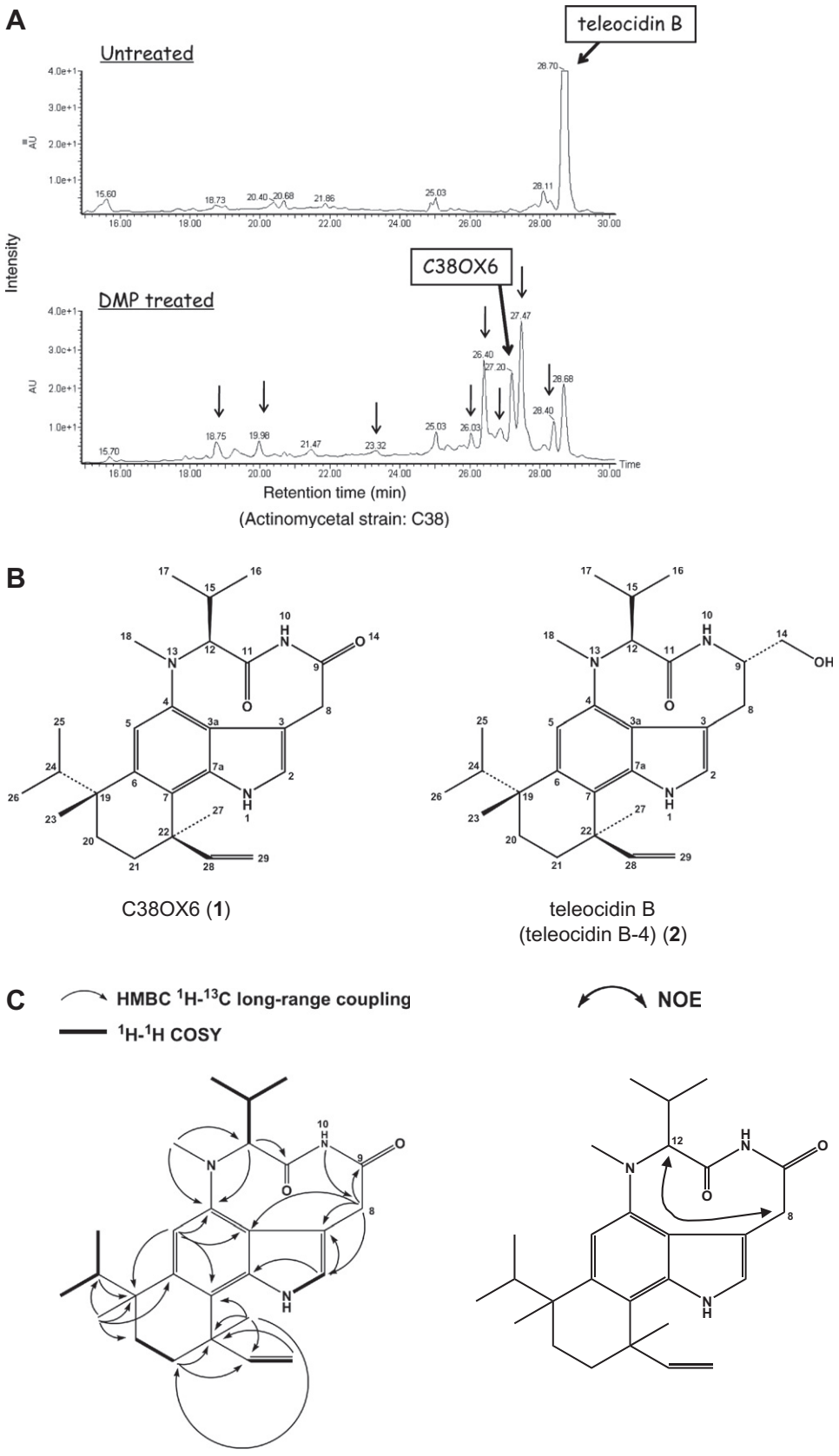

Figure 2. Structure elucidation of C38OX6 (**1**). (A) PDA chromatogram of DMP-treated or untreated broth extract of actinomycetal strain C38. Reaction and analysis conditions are as shown in Fig. 1B. Representative peaks of UNPs are indicated with arrows. (B) Structures of C38OX6 (**1**) and teleocidin B (**2**). (C) Structure elucidation of C38OX6 (**1**) by 2D-NMR spectroscopy.

2.2. Identification of C38OX6 (1) as XIAP inhibitor

Next, biological activities of each UNP were evaluated by several in-house assay systems. As a result, several UNPs showed biological activities such as apoptosis-inducing activity and migration inhibitory activity against cancer cells (data not shown). Among these active compounds, we focused on an UNP, C38OX6 (**1**), because it showed inhibitory activity against XIAP functions.

XIAP inhibits the enzymatic activity of caspase-3, resulting in cellular apoptosis suppression; therefore, to find candidate drug leads for tumor treatment, we searched for compounds in UNPs that could restore XIAP-suppressed caspase-3 activity. For this, we used the in vitro caspase de-repression assay using a fluorogenic peptide as a substrate.[10] As a result, one UNP, C38OX6, was found to overcome XIAP-mediated suppression of caspase-3. C38OX6 (**1**) was generated by the addition of DMP to the broth extracts of actinomycetal strain C38 (Fig. 2A). The preparation scheme of C38OX6 (**1**) is summarized in Figure S1.

Next we attempted to elucidate the structure of C38OX6 (**1**). From HRESI-MS measurements in combination with its ^{1}H and ^{13}C NMR data, the molecular formula of C38OX6 (**1**) was determined to be $C_{27}H_{37}N_3O_2$ (Found: 458.2783 $[M+Na]^+$, Calcd: 458.2783). The molecular formula and ^{1}H and ^{13}C NMR spectra indicated that the structure of C38OX6 (**1**) was quite similar to that of teleocidin B (**2**) (also known as teleocidin B-4 or olivoretin D)[11,12]; therefore, a structural study on C38OX6 (**1**) was performed by comparison with teleocidin B (**2**) (Fig. 2B). The molecular mass of C38OX6 (**1**) (435.60, $C_{27}H_{37}N_3O_2$) was 16 mass units smaller than that of teleocidin B (**2**) (451.64, $C_{28}H_{41}N_3O_2$), corresponding to the loss of CH_4 from teleocidin B (**2**). The UV/vis spectrum of C38OX6 (**1**) (λ_{max}^{MeOH} 232, 283 nm) was almost identical to that of teleocidin B (**2**) (λ_{max}^{MeOH} 231, 286 nm), indicating that they possess the same aglycone (indole).[11] The structure of C38OX6 (**1**) was mainly determined by NMR spectral analyses as follows. The ^{13}C NMR spectrum of C38OX6 (**1**) was quite similar to that of teleocidin B (**2**). Meanwhile, sp^3 methylene (δ 65.0, C-14) and sp^3 methine (δ 56.0, C-9) signals in teleocidin B (**2**) were not observed in C38OX6 (**1**) and a carbonyl carbon (δ 173.6), which does not exist in teleocidin B (**2**), was observed in C38OX6 (**1**). After establishing the direct connectivity between each proton and carbon by the HMQC spectrum, the partial structures in C38OX6 (**1**) were investigated by HMBC experiment. In the HMBC spectrum, ^{1}H–^{13}C long-range couplings from H-8 (δ 3.89 and δ 4.27, isolated methylene) to C-2 (δ 123.9), C-3 (δ 107.1), and C-9 (δ 173.6), and from 10-NH (δ 7.53) to C-8 (δ 38.9) were observed (Fig. 2C). Considering these findings and the molecular formula, the planar structure of C38OX6 (**1**) was determined as shown in Figure 2B. The ^{1}H and ^{13}C NMR spectral data for C38OX6 (**1**) are summarized in Table 1.

The DMP-untreated (control) broth extract of actinomycetal strain C38 contained a significant amount of teleocidin B (**2**), which was decreased in the DMP-treated broth extract (Fig. 2A). This finding raised the possibility that teleocidin B (**2**) was converted into C38OX6 (**1**), at least partly, by the DMP-mediated reaction. To confirm this possibility, purified teleocidin B (**2**) was suspended in CH_2Cl_2 and stirred with DMP in the same manner as the crude broth extract. As a result, C38OX6 (**1**) was detected in this reaction mixture by the LC–PDA–MS system (Fig. S2), indicating that C38OX6 (**1**) was the product generated by DMP-mediated oxidation of C-9 (sp^3 methine) in teleocidin B (**2**) to a carbonyl carbon. C38OX6 (**1**) has three chiral centers (C-12, C-19, and C-22). Since the stereochemistry of teleocidin B (**2**) has already been determined (9*S*, 12*S*, 19*R*, and 22*R*), the stereochemistry of C38OX6 (**1**) was also determined to be [12*S*, 19*R*, and 22*R*] (Fig. 2B).

Teleocidin B (**2**) and its analogs are known to exist mainly in two stable conformers in solution, the *cis* amide in a twist conformation and the *trans* amide in a sofa conformation[13]; however, C38OX6 (**1**) exists in only one conformer in $CHCl_3$ at room temperature. As NOE between H-8 (δ 4.27) and H-12 (δ 4.66) was observed (Fig. 2C), the conformation of C38OX6 (**1**) was determined to be a twist.

2.3. C38OX6 (1) restores XIAP-suppressed enzymatic activity of caspase-3 by inhibition of direct protein–protein interaction

As shown in Figure 3A, C38OX6 (**1**) restored XIAP-suppressed enzymatic activity of caspase-3 at 10 μM without affecting caspase-3 activity in the absence of XIAP. Meanwhile, we could not detect XIAP-inhibitory activity by teleocidin B (**2**), because teleocidin B (**2**) increased caspase-3 activity even in the absence of XIAP (data not shown).

Previous studies showed that XIAP inhibited the enzymatic activities of caspase-3 by direct binding[2]; therefore, we examined whether C38OX6 (**1**) interfered with the binding of XIAP to truncated active caspase-3 using an in vitro GST pull-down assay. Consistent with previous reports, GST-XIAP bound the active form of caspase-3, and this binding was inhibited by C38OX6 (**1**) at over 3 μM (Fig. 3B). This finding suggests that C38OX6 (**1**) interferes with the binding of XIAP to active caspase-3, thereby restoring XIAP-suppressed enzymatic activity of caspase-3. On the other

Table 1
^{13}C and ^{1}H NMR data of C38OX6 (**1**) and teleocidin B (**2**) in $CDCl_3$

Position	C38OX6 (**1**)[a]		Teleocidin B (**2**) (twist conformer)[b]	
	δ_C (ppm)	δ_H (ppm)	δ_C (ppm)	δ_H (ppm)
1		8.83 (1H, br s)		8.67 (1H, br s)
2	123.9	7.01 (1H, d, 1.3)	120.8	6.78 (1H, s)
3	107.1		114.1	
3a	117.3		116.8	
4	145.1		146.0	
5	107.4	6.50 (1H, s)	106.2	6.49 (1H, s)
6	139.7		138.5	
7	119.3		118.0	
7a	138.0		137.8	
8	38.9	3.89 (1H, d, 14.9)	33.7	3.01 (1H, dd, 3.6, 17.2)
		4.27 (1H, d, 14.9)		3.11 (1H, br d, 17.2)
9	173.6		56.0	4.33 (1H, m)
10		7.53 (1H, br s)		7.81 (1H, s)
11	174.5		174.8	
12	73.4	4.66 (1H, d, 10.1)	70.7	4.30 (1H, d, 10.1)
14			65.0	3.56 (1H, m)
				3.72 (1H, m)
15	28.8	2.63 (1H, m)	28.4	2.61 (1H, m)
16	19.9	0.80 (3H, d, 6.6)	19.6	0.68 (3H, d, 6.8)
17	21.8	0.96 (3H, d, 6.2)	21.5	0.91 (1H, d, 6.2)
18	33.0	2.81 (3H, s)	33.0	2.89 (3H, s)
19	40.7		40.0	
20	25.6	1.87*, 1.90* (2H)	24.9	1.4*, 1.9* (2H)
21	35.4	1.87*, 1.90* (2H)	34.8	1.4*, 1.9* (2H)
22	40.3		39.6	
23	29.7	1.31 (3H, s)	29.0	1.34 (3H, s)
24	38.5	2.22 (1H, m)	37.8	2.24 (1H, qq, 6.6, 6.8)
25	17.6	1.00 (3H, d, 6.8)	16.9	1.00 (3H, d, 6.8)
26	18.6	0.53 (3H, d, 6.8)	17.1	0.53 (3H, d, 6.6)
27	22.4	1.50 (3H, s)	21.7	1.50 (3H, s)
28	152.4	6.14 (1H, dd, 10.6, 17.7)	151.8	6.16 (1H, dd, 10.6, 17.6)
29	112.2	5.37 (1H, dd, 1.1, 10.6)	111.3	5.24 (1H, d, 10.6)
		5.43 (1H, dd, 1.1, 17.7)		5.40 (1H, d, 17.6)

Chemical shifts in ppm from TMS as internal standard.
* Obscured by overlapping signals.
[a] Twist (*cis* amide) conformer only (0.04 M, 25 °C).
[b] Twist (*cis* amide) conformer >90% (0.07 M, 24 °C).

hand, teleocidin B (**2**) did not inhibit the binding of GST-XIAP to active caspase-3 (Fig. S3).

2.4. Affinity of C38OX6 (1) for PKC isozymes

It was reported that naturally occurring teleocidin analogs potently bind to and activate protein kinase C (PKC) isozymes, resulting in tumor-promotion.[14,15] Because C38OX6 (**1**) is an analog of teleocidins, we examined whether C38OX6 (**1**) would bind to PKC isozymes. The affinity of C38OX6 (**1**) for each PKC isozyme was evaluated on the basis of inhibition of the specific binding of [^{3}H]phorbol 12,13-dibutyrate (PDBu) to each PKC C1 peptide.[16] As summarized in Table 2, the K_i values of C38OX6 (**1**) for conventional and novel PKC C1 domains were several hundred nanomolars, except that for the PKCδ C1B domain, whose K_i value is 87 nM (Table 2). Meanwhile, teleocidin B (**2**) showed much higher affinity for each PKC isozyme (e.g., K_i value for the PKCα C1A domain was 2.1 nM).

Table 2
K_i values for inhibition of the specific binding of [^{3}H]PDBu by C38OX6 (**1**)

PKC C1 peptides[a]	K_i (nM) C38OX6 (**1**)	K_d (nM) [^{3}H]PDBu[b]
α-C1A	600 (40)[c]	1.1
β-C1A	730 (50)	1.3
γ-C1A	660 (40)	1.5
δ-C1B	87 (5)	0.53
ε-C1B	480 (60)	0.81
η-C1B	360 (40)	0.45

[a] The C1A domains of conventional PKCs (α, β, and γ) are mainly involved in phorbol ester binding and translocation from the cytosol to plasma membrane (Refs. 27,28), whereas the C1B domains of novel PKCs (δ, ε, and η) play a critical role in translocation in response to the phorbor ester TPA (Ref. 29). Our binding data using PKC C1 peptides supports these data; the major binding sites of indolactam-V, the core structure of teleocidins, are the C1A domains of conventional PKCs and the C1B domains of novel PKCs (Ref. 30). Therefore, we used the C1A peptides of conventional PKCs and the C1B peptides of novel PKCs to evaluate the binding affinity for PKC isozymes of our compounds.
[b] Ref. 25.
[c] Standard deviation from triplicate experiments.

2.5. C38OX6 (1) sensitizes cancer cells to anticancer drugs

Previous studies showed that knockdown of XIAP using antisense oligonucleotides sensitized cancer cells to conventional anticancer drugs.[17] We also performed RNAi experiments to investigate whether the loss of XIAP could sensitize human cervical carcinoma HeLa cells to anticancer drugs. The successful knockdown of XIAP by siRNA was confirmed by immunoblotting (Fig. 4A). We confirmed that specific siRNA for XIAP sensitized HeLa cells to camptothecin or staurosporine (Fig. 4A). We therefore examined whether our XIAP inhibitor could also sensitize HeLa cells to camptothecin or staurosporine. As shown in Figure 4B, apoptosis induced by camptothecin or staurosporine was significantly promoted by co-treatment with C38OX6 (**1**) in HeLa cells. These results were further

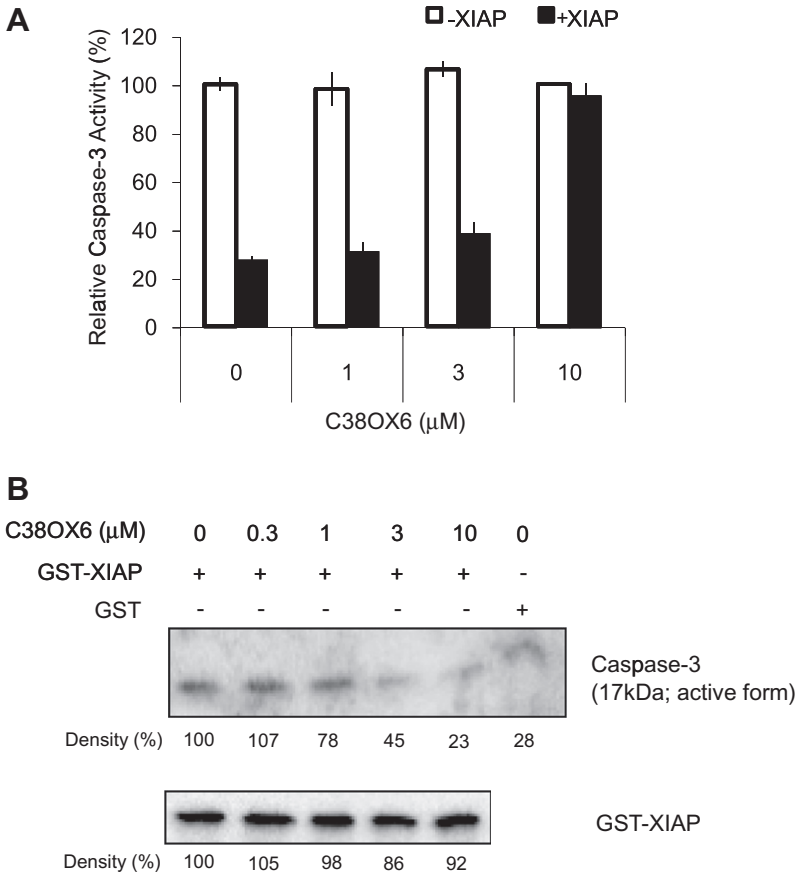

Figure 3. C38OX6 (**1**) interfered with the interaction between XIAP and caspase-3. (A) Effects of C38OX6 (**1**) on XIAP-suppressed caspase-3 activity. Recombinant active caspase-3 and C38OX6 (**1**) were mixed and incubated for 1 h at 37 °C in the presence or absence of GST-XIAP. Activity of caspase-3 was measured by the fluorogenic substrate Ac-DEVD-AFC. Values are the means of three independent determinations. Bars, SD. (B) Effects of C38OX6 (**1**) on the binding of XIAP to active caspase-3. Glutathione-Sepharose 4B beads, GST-XIAP (or GST), and active caspase-3 were incubated with C38OX6 (**1**) at 4 °C for 1 h. After washing, bound proteins were separated by SDS–PAGE followed by immunoblotting with anti-caspase-3 and anti-GST antibodies.

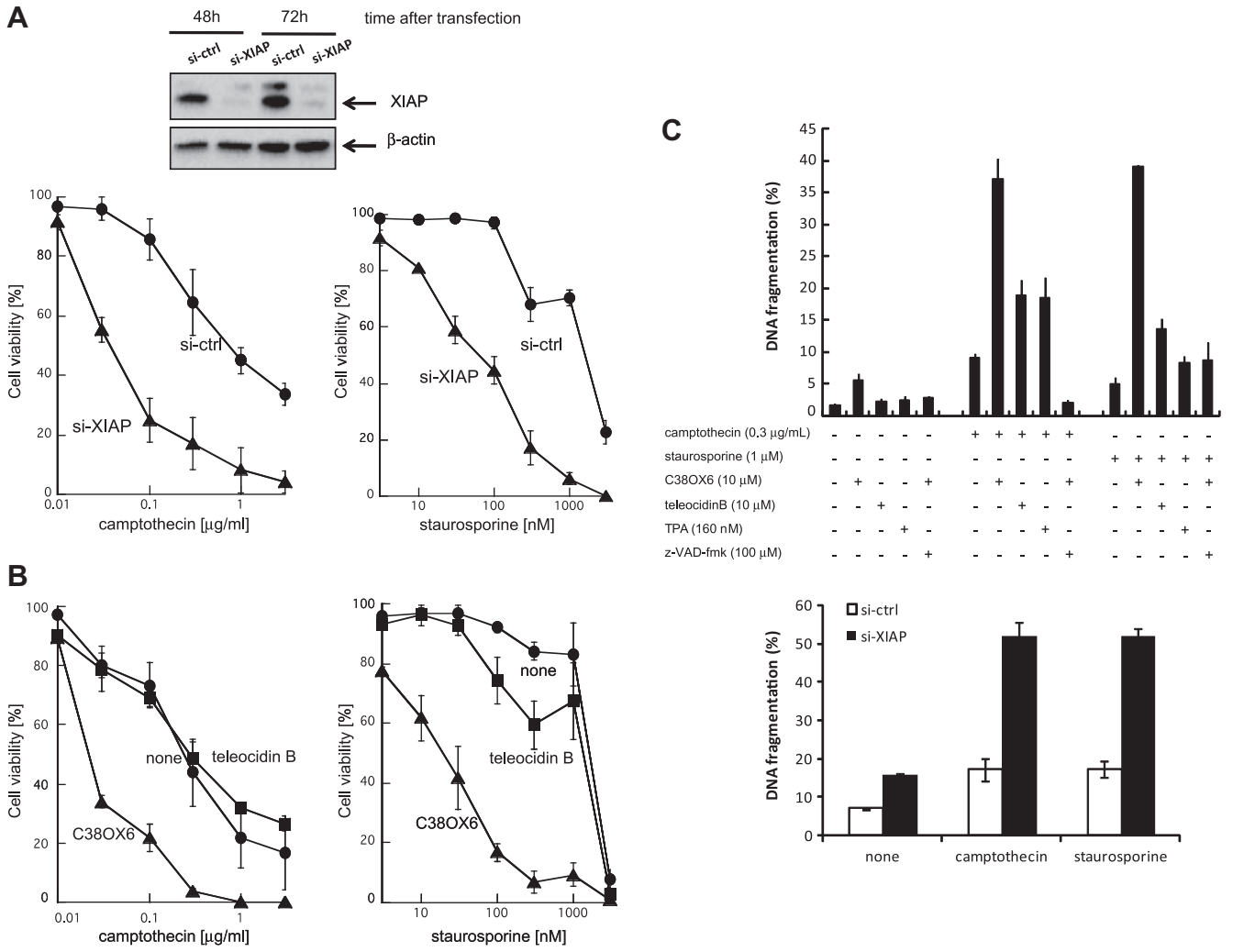

Figure 4. Effects of C38OX6 (**1**) on the sensitivity of cancer cells to anticancer drugs. (A) HeLa cells were transfected with XIAP or control siRNA 48 h before treatment of camptothecin. After 48 h of chemical treatment, cell viability was determined by trypan blue dye exclusion assay (bottom). Expression levels of XIAP and β-actin were examined by SDS–PAGE and immunoblotting (top). (B) HeLa cells were treated for 48 h with indicated concentrations of camptothecin or staurosporine in the presence or absence of test compounds (10 μM). Cell viability was determined by trypan blue dye exclusion assay. (C) HeLa cells were treated for 24 h with camptothecin (0.3 μg/mL) or staurosporine (1 μM) in the presence or absence of test compounds (top). HeLa cells were transfected with XIAP or control siRNA 48 h, followed by chemical treatment (bottom). Cell viability was determined by FACS analysis of DNA fragmentation of propidium iodide-stained nuclei (sub-G1 population was measured). Values are the means of three independent determinations. Bars, SD.

confirmed by propidium iodide-staining assay. As shown in Figure 4C, treatment of HeLa cells with C38OX6 (**1**) in the presence of camptothecin or staurosporine induced DNA fragmentation. This DNA fragmentation was suppressed by z-VAD-fmk, a pan-caspase inhibitor (Fig. 4C), indicating that C38OX6 (**1**) and the anticancer drug synergistically induced caspase-dependent apoptosis. To exclude the possibility that this apoptosis-enhancing effect of C38OX6 (**1**) is due to its weak but still retained PKC-binding ability, teleocidin B was examined for its ability to enhance anticancer drug-induced apoptosis. As shown in Fig. 4B and C, significant synergistic effects of teleocidin B with camptothecin or staurosporine were not observed in HeLa cells. In addition, 12-*O*-tetradecanoylphorbol-13-acetate (TPA), a phorbol ester-type tumor promoter that is known to activate PKC isozymes, did not sensitize HeLa cells to camptothecin and staurosporine at the effective dose range for PKC activation (Fig. 4C). These results suggest that C38OX6 (**1**) sensitized HeLa cells to anticancer drug-induced apoptosis, possibly due to its inhibitory effect on the anti-apoptotic function of XIAP, but not due to the activation of PKC.

3. Discussion

Historically, a number of bioactive natural products, such as antibiotics, antimalarials, immunosuppressants, and anticancer drugs, have revolutionized medicine. Indeed, among all available anticancer drugs from around the 1940s to 2006, 14% were natural products and 28% were natural product derivatives,[18] indicating that natural products and their derivatives are still high-quality sources for drug-lead discovery. Therefore, in the present study, we focused on the generation and use of a new chemical library consisting of chemically modified microbial metabolites which are referred to as 'Unnatural Natural Products (UNPs)'.

To generate the UNP library, we devised and developed a series of unique procedures via the addition of chemical reagents to crude microbial broth extracts combined with the detection and purification of reaction products (UNPs) by the LC–PDA–MS system. In these procedures, the reaction conditions of chemical conversion were quite important factors because each actinomycetal strain produces different secondary metabolites and some metabolites

are unstable at extreme temperature and pH. However, we established the broadly applicable reaction conditions after several examinations and, using our established method, generated an UNP library consisting of various types of compounds, such as trieneansamycin compounds, polyene-macrolide compounds, anthraquinone compounds and so on.

For the construction of chemical libraries consisting of natural product analogs, several approaches have been reported. One of the most intensively researched approaches is combinatorial biosynthesis, especially using modular polyketide synthases (PKSs) and non-ribosomal peptide synthetases (NRPSs), which are large multidomain enzymes sequentially condensing short fatty acids and α-amino acids, respectively.[19] For example, the 'Unnatural Natural Product library' consisting of polyketides was constructed by multiple genetic modifications of 6-deoxyerythronolide B synthase, the PKS, which produces the macrolide ring of erythromycin.[20] As another unique approach, genetic code reprogramming by a ribozyme-based de novo tRNA acylation system combined with a cell-free translation system enabled the generation of natural product-like non-standard peptides.[21] Compared with the above approaches, chemical conversion is a simple and somewhat classical approach for generating natural product analogs, and is applicable to various types of compounds. In addition, the combination of chemical conversion of microbial metabolites in crude broth extracts and purification by the LC–PDA–MS system enabled us to effectively generate the UNP library.

In addition to developing the UNP library, we also aimed to utilize it. In the course of screening for unique bioactive compounds by several in-house assay systems, C38OX6 (**1**) was identified as a new inhibitor of XIAP. C38OX6 (**1**) abrogated the interaction between XIAP and active caspase-3, thereby restoring XIAP-suppressed enzymatic activity of caspase-3 (Fig. 3A and B). Similarly, two types of compounds, aryl sulfonamide compounds and polyphenylurea compounds, were previously reported as XIAP inhibitors which interfered with the binding of XIAP to caspase-3.[10,22] In the same manner as treatment of polyphenylurea compounds, C38OX6 (**1**) sensitized cancer cells to anticancer drugs (Fig. 4B and C), and the effective concentration of C38OX6 (**1**) in cells was approximately the same degrees as in aryl sulfonamide compounds and polyphenylurea compounds.

We confirmed that C38OX6 (**1**) was a reaction product generated by DMP-mediated conversion of teleocidin B (**2**). As teleocidin B (**2**) is known to exhibit tumor-promoting activity by potent binding to and activation of several PKC isozymes,[15] it is noteworthy that the affinity of C38OX6 (**1**) for each PKC isozyme was significantly decreased as compared to that of telocidin B (K_i value for the PKCα C1A domain was 2.1 nM; Table 2). A methine carbon (C-9) linked to a hydroxymethyl group in teleocidin B (**2**) was changed into a carbonyl carbon in C38OX6 (**1**). These findings are consistent with a previous report that a free hydroxyl group at C-14 was important for the tumor-promoting activity of teleocidin B (**2**).[23] Although the affinity of C38OX6 (**1**) for conventional and novel PKC isozymes was much lower than that of teleocidin B (**2**), we could not completely reject the involvement of some PKC isozymes in sensitization to anticancer drugs by C38OX6 (**1**). However, potent PKC activators, teleocidin B (**2**) and TPA, failed to sensitize HeLa cells to anticancer drugs at effective concentrations for PKC activation (Fig. 4B and C). These results strongly suggest that C38OX6 (**1**) sensitizes cancer cells to anticancer drugs by its XIAP-inhibitory activity, but not in a PKC-dependent manner. After structure–activity relationship study of C38OX6 (**1**) for complete removal of PKC activation and increased XIAP inhibition, a new class of anticancer drugs that inhibit XIAP will be developed.

In the DMP-treated broth extract of actinomycetal strain C38, several teleocidin-like UNPs judged from their physico-chemical properties were detected. However, all tested teleocidin-like UNPs other than C38OX6 (**1**) did not inhibit the anti-apoptotic function of XIAP like teleocidin B (**2**) (data not shown).

This study suggests that UNPs are superior to their original natural products in cases such as C38OX6 (**1**), and generation of the UNP library is a promising approach to increase the diversity of sources and obtain unique bioactive compounds for drug development.

4. Conclusion

In the present study, we generated a new chemical library consisting of chemically modified microbial metabolites which are referred to as 'Unnatural Natural Products (UNPs)' through a series of procedures via chemical conversion of microbial metabolites in crude broth extracts followed by purification of reaction products with the LC–PDA–MS system. Using our UNP library, we discovered an XIAP inhibitor, C38OX6 (**1**), which interfered with the binding of XIAP to caspase-3, thereby restoring XIAP-suppressed enzymatic activity of caspase-3 in vitro. Furthermore, C38OX6 (**1**) was found to sensitize cancer cells to anticancer drugs.

5. Material and methods

5.1. General experimental procedure. Fermentation of actinomycetes and preparation of broth extracts, preparation of C38OX6 (1) and teleocidin B (2)

See 'Supplementary data' section.

5.2. Cell culture

Human cervical carcinoma HeLa cell line was cultured in DMEM containing 8% fetal bovine serum, 2.5 g/L sodium bicarbonate, penicillin G (100 units/mL), and kanamycin (0.1 g/L).

5.3. Caspase de-repression assay

The caspase de-repression assay was performed as described previously with several modifications.[10] Briefly, cDNA of full-length human XIAP was cloned from HeLa cells and inserted as an EcoRI fragment into the pGEX-2T vector (GE Healthcare, NJ), and recombinant GST-XIAP was produced in *Escherichia coli* and purified. Recombinant human active caspase-3 (0.01 unit; Enzo Life Sciences, NY), recombinant GST-XIAP (100 ng), a test compound, and the fluorogenic tetrapeptide substrate acetyl-Asp-Glu-Val-Asp-[7-amino-4-trifluoromethylcoumarin](Ac-DEVD-AFC, 10 μM; Enzo Life Sciences) were mixed and incubated in caspase assay buffer (20 mM Tris, 100 mM NaCl, 10 mM DTT, and 3% glycerol, pH 7.5) for 1 h at 37 °C after preincubation for 30 min at 30 °C without the fluorogenic substrate. The activity of caspase-3 was measured by monitoring the cleavage of Ac-DEVD-AFC using a spectrofluorometric plate reader at excitation and emission wavelengths of 405 and 510 nm, respectively.

5.4. GST pull-down assay

Recombinant GST-XIAP (or GST) (1 μg), recombinant human active caspase-3, and 10 μL Glutathione-Sepharose 4B beads (GE Healthcare) were incubated at 4 °C for 1 h in 1 mL of caspase assay buffer in the presence or absence of a test compound. After three washes, the bound proteins were separated by SDS–PAGE, followed by immunoblotting with the primary antibodies against caspase-3 (sc-7148; Santa Cruz, CA) or GST (sc-138; Santa Cruz).

5.5. [^{3}H]PDBu-binding assay

The binding of [^{3}H]PDBu to PKC C1 peptides was evaluated using the procedure of Sharkey and Blumberg[24] with modifications

as reported previously[25] with 50 mM Tris-maleate buffer (pH 7.4 at 4 °C), 10–40 nM PKC C1 peptide, 20 nM [^{3}H]PDBu (16.3 Ci/mmol), 50 μg/mL 1,2-dioleoyl-*sn*-glycero-3-phospho-L-serine, 3 mg/mL bovine γ-globulin, and various concentrations of inhibitor. Binding affinity was evaluated on the basis of the concentration required to cause 50% inhibition of the specific binding of [^{3}H]PDBu, IC_{50}, which was calculated with PriProbit 1.63 software.[26] The inhibition constant K_i was calculated using the method of Sharkey and Blumberg.[24] Although we used each PKC C1 peptide in the range 10–40 nM, the concentration of the properly folded peptide was estimated to be about 3 nM based on the B_{max} values of the Scatchard analyses reported previously[25]; therefore, the concentration of free PDBu will not markedly vary over the dose–response curve.

5.6. Knockdown of XIAP using siRNA

siRNA for XIAP (HSS100565) and the control (12935-112) were purchased from Invitrogen (CA), and were transfected into cells using Lipofectamine RNAi MAX (13778150, Invitrogen). After 48 h of transfection of each siRNA, test compounds were treated, followed by cell death assay. Expression levels of XIAP and β-actin (loading control) were examined by SDS–PAGE and immunoblotting with the primary antibodies against XIAP (610762; BD Biosciences Pharmingen, CA) and β-actin (A5316; Sigma–Aldrich, MO).

5.7. Cell death assay

HeLa cells were seeded at 2×10^4 cells/well in 48-well plates and cultured overnight. The cells were treated with various concentrations of test compounds for 48 h. Cell viability was counted by trypan blue dye exclusion assay (Fig. 4A and B). HeLa cells were seeded at 1×10^5 cells/well in 12-well plates and cultured overnight. The cells were treated with various concentrations of test compounds for 24 h. Subsequently, cells were stained with propidium iodide, followed by determination of cell death by fluorescence-activated cell sorting (FACS) analysis (Epics Altra; Beckman Coulter, CA) of DNA fragmentation of propidium iodide-stained nuclei (sub-G1 population was measured) (Fig. 4C). z-VAD-fmk was purchased from Sigma–Aldrich (MO).

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2011.05.009. These data include MOL files and InChiKeys of the most important compounds described in this article.

References and notes

1. LaCasse, E. C.; Mahoney, D. J.; Cheung, H. H.; Plenchette, S.; Baird, S.; Korneluk, R. G. *Oncogene* **2008**, *27*, 6252.
2. Deveraux, Q. L.; Takahashi, R.; Salvesen, G. S.; Reed, J. C. *Nature* **1997**, *388*, 300.
3. Deveraux, Q. L.; Roy, N.; Stennicke, H. R.; Van Arsdale, T.; Zhou, Q.; Srinivasula, S. M.; Alnemri, E. S.; Salvesen, G. S.; Reed, J. C. *EMBO J.* **1998**, *17*, 2215.
4. Deveraux, Q. L.; Leo, E.; Stennicke, H. R.; Welsh, K.; Salvesen, G. S.; Reed, J. C. *EMBO J.* **1999**, *18*, 5242.
5. Harlin, H.; Reffey, S. B.; Duckett, C. S.; Lindsten, T.; Thompson, C. B. *Mol. Cell. Biol.* **2001**, *21*, 3604.
6. Li, J. W.; Vederas, J. C. *Science* **2009**, *325*, 161.
7. Feher, M.; Schmidt, J. M. *J. Chem. Inf. Comput. Sci.* **2003**, *43*, 218.
8. Abel, U.; Koch, C.; Speitling, M.; Hansske, F. G. *Curr. Opin. Chem. Biol.* **2002**, *6*, 453.
9. Liu, X.; Ashforth, E.; Ren, B.; Song, F.; Dai, H.; Liu, M.; Wang, J.; Xie, Q.; Zhang, L. *J. Antibiot.* **2010**, *63*, 415.
10. Schimmer, A. D.; Welsh, K.; Pinilla, C.; Wang, Z.; Krajewska, M.; Bonneau, M. J.; Pedersen, I. M.; Kitada, S.; Scott, F. L.; Bailly-Maitre, B., et al *Cancer Cell* **2004**, *5*, 25.
11. Sakai, S.; Aimi, N.; Yamaguchi, K.; Hitotsuyanagi, Y.; Watanabe, C.; Yokose, K.; Koyama, Y.; Shudo, K.; Itai, A. *Chem. Pharm. Bull.* **1984**, *32*, 354.
12. Hitotsuyanagi, Y.; Fujiki, H.; Suganuma, M.; Aimi, N.; Sakai, S.; Endo, Y.; Shudo, K.; Sugimura, T. *Chem. Pharm. Bull.* **1984**, *32*, 4233.
13. Irie, K.; Isaka, T.; Iwata, Y.; Yanai, Y.; Nakamura, Y.; Koizumi, F.; Ohigashi, H.; Wender, P. A.; Satomi, Y.; Nishino, H. *J. Am. Chem. Soc.* **1996**, *118*, 10733.
14. Fujiki, H.; Suganuma, M.; Matsukura, N.; Sugimura, T.; Takayama, S. *Carcinogenesis* **1982**, *3*, 895.
15. Fujiki, H.; Sugimura, T. *Adv. Cancer Res.* **1987**, *49*, 223.
16. Irie, K.; Oie, K.; Nakahara, A.; Yanai, Y.; Ohigashi, H.; Wender, P. A.; Fukuda, H.; Konishi, H.; Kikkawa, U. *J. Am. Chem. Soc.* **1998**, *120*, 9159.
17. Hu, Y.; Cherton-Horvat, G.; Dragowska, V.; Baird, S.; Korneluk, R. G.; Durkin, J. P.; Mayer, L. D.; LaCasse, E. C. *Clin. Cancer Res.* **2003**, *9*, 2826.
18. Newman, D. J.; Cragg, G. M. *J. Nat. Prod.* **2007**, *70*, 461.
19. Weissman, K. J.; Müller, R. *Chembiochem* **2008**, *9*, 826.
20. McDaniel, R.; Thamchaipenet, A.; Gustafsson, C.; Fu, H.; Betlach, M.; Ashley, G. *Proc. Natl. Acad. Sci. U.S.A.* **1999**, *96*, 1846.
21. Murakami, H.; Ohta, A.; Ashigai, H.; Suga, H. *Nat. Methods* **2006**, *3*, 357.
22. Wu, T. Y.; Wagner, K. W.; Bursulaya, B.; Schultz, P. G.; Deveraux, Q. L. *Chem. Biol.* **2003**, *10*, 759.
23. Horiuchi, T.; Fujiki, H.; Suganuma, M.; Hakii, H.; Nakayasu, M.; Hitotsuyanagi, Y.; Aimi, N.; Sakai, S.; Endo, Y.; Shudo, K., et al *Gann* **1984**, *75*, 837.
24. Sharkey, N. A.; Blumberg, P. M. *Cancer Res.* **1985**, *45*, 19.
25. Shindo, M.; Irie, K.; Nakahara, A.; Ohigashi, H.; Konishi, H.; Kikkawa, U.; Fukuda, H.; Wender, P. A. *Bioorg. Med. Chem.* **2001**, *9*, 2073.
26. Sakuma, M. *Appl. Entomol. Zool.* **1998**, *33*, 339.
27. Raghunath, A.; Ling, M.; Larsson, C. *Biochem. J.* **2003**, *370*, 901.
28. Quest, A. F. G.; Bell, R. M. *J. Biol. Chem.* **1994**, *269*, 20000.
29. Szallasi, Z.; Bogi, K.; Gohari, S.; Biro, T.; Acs, P.; Blumberg, P. M. *J. Biol. Chem.* **1996**, *271*, 18299.
30. Masuda, A.; Irie, K.; Nakagawa, Y.; Ohigashi, H. *Biosci., Biotechnol., Biochem.* **2002**, *66*, 1615.