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Castalagin and vescalagin purified from leaves of *Syzygium samarangense* (Blume) Merrill & L.M. Perry: Dual inhibitory activity against PARP1 and DNA topoisomerase II

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ABSTRACT

Inhibition of poly(ADP-ribose) polymerase 1 (PARP1) is one of the most promising strategies for cancer chemotherapy, and a number of inhibitors possessing nicotinamide-like structures are being developed. To discover new types of PARP1 inhibitors, we screened a large number of substances of plant origin and isolated two inhibitory substances from the leaves of *Syzygium samarangense* (Blume) Merrill & L.M. Perry. The inhibitory substances were identified as vescalagin and its epimer castalagin by analyses using nuclear magnetic resonance and mass spectrometry. The IC_{50} of purified vescalagin and castalagin for PARP1 inhibition were 2.67 and 0.86 μM, respectively. Unlike most of synthetic PARP1 inhibitors, castalagin showed a mixed type inhibition, of which Ki was 1.64 μM. When SH-SY5Y cells were treated with these ellagitannins at concentrations of less than 5 μM, cellular poly(ADP-ribosyl)ation was obviously attenuated. Castalagin and vescalagin also possessed inhibitory activity against DNA topoisomerase II, implying that they function as dual inhibitors in cells.

1. Introduction

Poly(ADP-ribosyl)ation (PARylation) is catalyzed by poly(ADP-ribose) polymerase (PARP), which successively transfers ADP-ribose moieties of NAD^+ to acceptor proteins and thereby forms poly(ADP-ribose) (PAR) chains that are covalently bound to acceptor proteins [1]. Among PARP family members, the first discovered, most abundant, and best-studied isoform is PARP1 (EC 2.4.2.30). The PARP1 molecule is structurally divided into an N-terminal DNA binding domain, a central automodification (autoPARylation) domain, and a C-terminal catalytic domain [2, 3]. The DNA binding domain includes three zinc-finger motifs that facilitate binding of the enzyme to single-stranded DNA break and/or double-stranded DNA breaks [2, 4]. Upon binding to the DNA break sites, PARP1 is activated and actively PARylates closely located proteins and the enzyme itself (so-called autoPARylation). During autoPARylation, specific glutamic acid and aspartic acid residues distributed in the automodification domain become the targets of PARylation [2, 3]. In addition, this domain has a breast cancer susceptibility protein C terminus motif, which is required for protein-protein interaction [3]. The third motif is the C-terminal catalytic domain, which contains the NAD^+ binding site and is the most conserved domain across the PARP family [2, 3].

PARP1 is involved in several cellular events such as DNA repair [5, 6] and transcriptional regulation [7, 8]. There are numerous reports that examine the role of PARP1 in the DNA repair process, which is due to the close relationship between the occurrence of DNA lesions and PARP1 activation [5, 6]. Exposing cells to oxidative stress, genotoxic agents, or irradiation causes DNA injuries to a variety of degrees. Damaged DNA is repaired through one of the following pathways, excision repair/single-stranded DNA breaks repair, non-homologous end joining, or homologous recombination (HR), depending on the damage type. Because PARP1 is closely involved in all repair pathways *via* interaction with DNA repair-related factors and/or PARylation of them [5, 6], combinations of PARP1 inhibitors with DNA damaging agents

Abbreviations: PARP, poly(ADP-ribose) polymerase; PAR, poly(ADP-ribose); PARylation, poly(ADP-ribosyl)ation; PARylate, poly(ADP-ribosyl)ate; ADP-ribose, ADPR; nicotinamide, Nam; CAS, castalagin; VES, vescalagin; DNA topoisomerase II, Top2

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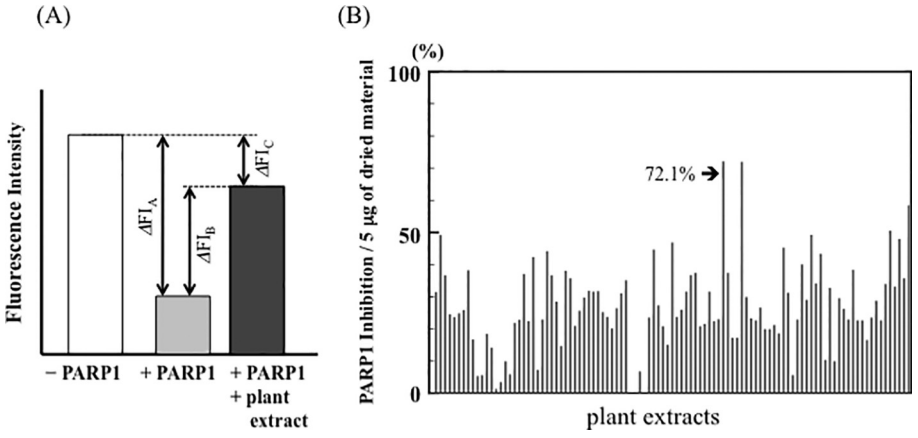

Fig. 1. Screening assay of plant extracts for PARP1 inhibitory activity.
(A) The PARP1 inhibitory activities of plant extracts were determined on the basis of the amount of unreacted NAD^+ after PARylation as described in Section 2.2. As a control, a PARP1-free reaction was carried out in parallel. After terminating the reactions, the residual NAD^+ in the reaction mixture was chemically converted to a fluorescent compound, and its fluorescence intensity (FI) was measured. ΔFI_A = [(FI of PARP1-free reaction mixture) – (FI of complete reaction mixture)], ΔFI_B = [(FI of complete reaction mixture with a plant extract) – (FI of complete reaction mixture)], and ΔFI_C = [(FI of PARP1-free reaction mixture) – (FI of complete reaction mixture with a plant extract)] were calculated, respectively. PARP1 inhibitory activity of a plant extract was estimated as degree of PARP1 inhibition (%): $(\Delta FI_B/\Delta FI_A) \times 100$ (%).
(B) One hundred sixty-two extract were prepared from various plant substances and stored at −30 °C as described in Section 2.4. The stock solutions were diluted 30-fold with DMSO, and 2 μL aliquots (containing 6.7 μg of dried materials) were used for the PARP1 inhibition assay in duplicate. When the inhibition (%) was undetectable or minimal, the inhibition assay was repeated using 10 × diluted extract (containing 20 μg of the dried materials). The values obtained from the above numerical formula of $(\Delta FI_B/\Delta FI_A) \times 100$ (%) shown in (A) were calibrated to values per 5 μg of dried materials as shown. In this figure, PARP1 inhibition values of 103 samples are displayed and another 59 samples showing extremely low or undetectable inhibitions were omitted. The leaf extract of *S. samarangens* showed the highest inhibition of 72.1%.

such as genotoxic agents and ionizing radiation may be potential as a strategy in cancer chemotherapy [9, 10]. Moreover, in the HR pathway, tumor suppressor proteins BRCA1/2 (breast cancer susceptibility gene 1 and 2) as well as PARP1 are key players to accomplish repair of double-stranded DNA breaks [11]. In this context, a finding that PARP1 inhibition can induce the cell death of *BRCA1/2*- deficient cancer cell lines [12, 13] suggested the effectiveness of PARP1 inhibitors as a monotherapy of *BRCA*-mutated cancers [14]. Taken together, these reports have demonstrated that the targeting of PARP1 could be greatly valuable as a cancer treatment [15].

To date, a number of PARP1 inhibitors have been developed [16–19], and one of them, olaparib (AZD2281), has been approved as an anticancer drug against a certain *BRCA*-mutated advanced ovarian cancer by the United States Food and Drug Administration [20]. Most of those synthetic PARP1 inhibitors were designed as compounds that have a nicotinamide (Nam)-like structure, and they were expected to compete with NAD^+ to bind to the catalytic domain of PARP1 [16–19]. However, because of the similarities of the catalytic domains among PARP family members [2, 3], those inhibitors showed high binding affinity to other PARP isoforms [21, 22]. On the other hand, a number of natural substances having PARP1 inhibitory activity have been reported [23], and some of them have been proven to possess potent inhibitory activity [24–28]. These discoveries encouraged us to find new potent PARP1 inhibitors of plant origin.

In this study, we demonstrate that castalagin (CAS) and vescalagin (VES) isolated from the leaves of *Syzygium samarangense* (Blume) Merr. & L.M. Perry (*S. samarangense*), inhibit PARP1 *in vitro* in a mixed type-dependent fashion. They could also attenuate cellular PARylation at a μM-level. In addition, we demonstrate that CAS and VES have inhibitory activity against DNA topoisomerase II (Top2) as reported in previous studies [29, 30]. We will discuss the biological significance of dual inhibition against PARP1 and Top2.

2. Materials and methods

2.1. Reagents and enzymes

Nicked DNA (designated as activated DNA) was prepared according to a previous method [31]. Briefly, calf thymus DNA was partially digested with DNase I, and the nicked DNA was purified by sequential treatment with phenol, chloroform, and ethyl alcohol. Purified bovine thymus PARP1 was obtained as described in a previous report [32]. PARP inhibitors olaparib [16] and 3-aminobenzamide (3-AB) [33] were purchased from AdooQ Bioscience (Canada) and Sigma-Aldrich Japan (Japan), respectively. Gallic acid was obtained from Tokyo Chemical Industry Co., Ltd. (Japan). Ellagic acid, DL-catechin, and tannic acid were obtained from Wako Pure Chemical Industries Ltd., Japan.

Goat polyclonal anti-mouse PARP1 (anti-PARP1) was obtained from R&D Systems, Inc. Mouse monoclonal anti-human Hsp90 (anti-Hsp90) was purchased from Enzo Life Sciences, Inc. Rabbit polyclonal anti-poly (ADP-ribose) (anti-PAR) was prepared as described previously [34]. The horseradish peroxidase (HRP)-conjugated rabbit polyclonal anti-mouse IgG and the HRP-conjugated rabbit polyclonal anti-goat IgG antibodies were purchased from Jackson ImmunoResearch Laboratories Inc., USA. A kit of molecular weight marker proteins for electrophoresis was obtained from Dynamarker, BioDynamics Laboratory Inc., Japan. All other chemicals used in this study were of analytical grade.

2.2. PARP1 inhibition assay

The PARP1 inhibitory activity of test substances such as plant extracts or purified products was measured according to a previous method with slight modifications [35]. Briefly, the reaction mixture containing 50 mM Tris − HCl buffer, pH 8.0, 10 mM $MgCl_2$, 2 μg/mL of purified PARP1, 15 ⌈g/mL of activated DNA, 10 ⌈M NAD^+, an appropriate amount of a test substance in a total volume of 125 μL was incubated at 25 °C for 30 min. In parallel, as positive and negative controls, PARP1 inhibition assays without either PARP1 or the specimens were carried out. The reactions were terminated by additions of 50 μL aliquots of 2 M KOH and 20% (V/V) acetophenone, respectively. After chilling at 4 °C for 10 min in the dark, 225 μL aliquots of 88% (V/V) formic acid was added to the mixture and heated at 110 °C for 5 min in the dark. The residual NAD^+ in the reaction mixture was converted to a fluorescent derivative through these processes [35]. After chilling, the fluorescence intensity (FI) of the mixed solution was measured with excitation at 360 nm and emission at 445 nm. For convenience, the inhibiting activity of the specimen was estimated as the degree of PARP1 inhibition (%) = $[\Delta FI_B/\Delta FI_A] \times 100\%$ as shown in the scheme (Fig. 1-A).

2.3. DNA topoisomerase II inhibition assay

The inhibitory activities of the test substances against DNA topoisomerase II (Top2) were measured using a human recombinant Top2