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Selective inhibitors of terminal deoxyribonucleotidyltransferase (TdT): Baicalin and genistin

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Abstract

Studies of mammalian terminal deoxyribonucleotidyltransferase (TdT) are facilitated by use of inhibitors that selectively knock down the activity of the enzyme. We have screened for selective inhibitors of TdT and identified a natural compound with this property in the Japanese vegetable, *Arctium lappa*. The compound has little effect on the activities of mammalian DNA polymerases, such as α, β, δ or λ polymerase, and prokaryotic DNA polymerases, such as Taq DNA polymerase, T4 DNA polymerase and Klenow fragment. H^1- and C^{13}-NMR spectroscopic analyses showed the compound to be baicalin, a compound previously reported as an anti-inflammatory or antipyretic agent. The IC_{50} value of baicalin to TdT was 18.6 μM. We also found that genistin, a baicalin derivative known to be antimutagenic, more selectively inhibited TdT activity than baicalin, although its IC_{50} value was weaker (28.7 μM). Genistin and baicalin also inhibited the activity of truncated TdT (the so-called pol β core domain) in which the BRCT motif was deleted in its N-terminal region. In kinetic analyses, inhibition by either genistin or baicalin was competitive with the primer and non-competitive with the dNTP substrate. The compounds may, therefore, bind directly to the primer-binding site of TdT and simultaneously disturb dNTP substrate incorporation into the primer. Genistin and baicalin should prove to be useful agents for studying TdT.

Keywords: Terminal deoxyribonucleotidyltransferase (TdT); TdT inhibitor; *Arctium lappa*; Baicalin; Genistin

1. Introduction

We have carried out a systematic search for natural compounds that selectively inhibit eukaryotic DNA polymerases, with the ultimate aim of using these compounds as tools and molecular probes to distinguish DNA polymerases and to clarify their in vivo biological functions (summarized in reference No. [1,2]). In this study, we describe newly found compounds that selectively inhibit the activity of the unique DNA polymerase, terminal deoxyribonucleotidyltransferase (TdT).

TdT has a BRCA-1 C-terminal (BRCT) domain in the N-terminal region and a pol β-like core domain that is required for DNA polymerase catalysis in the C-terminal region. In vitro, TdT catalyses the polymerization of deoxyribonucleotides to the 3′ hydroxyl end of single-stranded DNA in the absence of a DNA template [3]. TdT participates in the diverse repertoire of the immune system by adding nucleotides (N regions) to junctions of DNA segments assembled during V(D)J recombination [4–7]. TdT also participates in non-homologous end joining (NHEJ), a central part of the system of double-stranded DNA break repair [8]. TdT-deficient mice display a complex range of phenotypes [5,6,9–11]. An alternative approach for investigation of the role of TdT is through use of compounds that selectively inhibit the action of the enzyme. For this reason,

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we have performed a screen for selective inhibitors of TdT, concentrating on compounds that affect human TdT.

As we report here, a large number of natural resources have been tested in our search for compounds that selectively inhibit TdT activity but not those of other DNA polymerases. We eventually found a candidate, subsequently identified as baicalin, in a water-soluble fraction extracted from *Arctium lappa*, a Japanese vegetable that is commonly known as gobo. Baicalin, and a derivative called genistin, did not show complete specificity for TdT but were highly selective for the polymerase. These compounds may provide an alternative approach for investigation of the roles of TdT.

2. Materials and methods

2.1. Materials

Nucleotides and chemically synthesized DNA template-primers, such as poly(dA), poly(rA), and oligo(dT)$_{12-18}$ and [^{3}H]dTTP (43 Ci/mmol), were purchased from Amersham Biosciences (Buckinghamshire, UK). All other reagents were of analytical grade and were purchased from Wako Pure Chemical Industries Ltd. (Osaka, Japan).

2.2. Enzymes

DNA polymerase α (pol α) was purified from calf thymus by immunoaffinity column chromatography [12]. Recombinant rat DNA polymerase β (pol β) was purified from *Escherichia coli* as described by Date et al. [13]. Recombinant human terminal deoxyribonucleotidyltransferase (TdT) and truncated TdT (pol β-like core domain) were purified from *Escherichia coli* as described by Ibe et al. [3]. Pol δ was purified from calf thymus [14]. The cDNA encoding human pol λ was generated by RT-PCR using the primers L-F1 (5′-GCAGAATTCATGGATCCCAGGGG-TATCTTGAAG-3′) and L-R1 (5′-GTTCTCGAGCCAGT-CCCGCTCAGCAGGTTCTCG-3′). The cDNA was cloned into the expression vector pET-28b (Novagen), and the *E. coli* BL21 (DE3) strain was transformed with the vector. Histidine-tagged enzyme was expressed according to the supplier's manual (Novagen) and purified using Ni-NTA Resin (Invitrogen). The Klenow fragment of pol I was purchased from Worthington Biochemical Corp. (Freehold, NJ). Taq DNA polymerase and T4 DNA polymerase were purchased from Takara (Tokyo, Japan).

2.3. TdT and DNA polymerase assays

The reaction mixtures for pol α, β, δ, λ and prokaryotic DNA polymerases were described previously [15]. The substrates of the DNA polymerases used were poly(dA)/oligo(dT)$_{12-18}$ and dTTP as DNA template-primer and nucleotide substrate, respectively. The substrates of terminal deoxyribonucleotidyltransferase used were oligo(dT)$_{12-18}$ (3′-OH) and dTTP as primer and nucleotide substrate, respectively.

Baicalin, baicalin hydrate, baicalein and genistin, purchased from Wako Pure Chemical Industries, Ltd. (Tokyo, Japan), were dissolved in water at various concentrations. Four microliters of the samples were mixed with 16 μl of each enzyme (final concentration 0.05 units) in 50 mM Tris–HCl (pH 7.5) containing 1 mM dithiothreitol, 50% glycerol, and 0.1 mM EDTA and kept at 0 °C for 10 min. Eight microliters of inhibitor–enzyme mixture were added to 16 μl of each of the standard enzyme reaction mixtures, and incubation was carried out at 37 °C for 60 min, except for Taq DNA polymerase which was incubated at 74 °C for 60 min. The level of enzyme activity in the absence of the inhibitor was designated as 100%; residual activities at each concentration of inhibitor were determined as percentages of this value. One unit of DNA polymerase activity was defined as the amount of enzyme that catalyzed the incorporation of 1 nmol of dTTP into the synthetic DNA template-primers [i.e., poly(dA)/oligo(dT)$_{12-18}$, A/T=2/1] in 60 min at 37 °C under the normal reaction conditions for each enzyme [15].

3. Results and discussion

3.1. Isolation of baicalin

As described briefly in the Introduction, we screened for terminal deoxyribonucleotidyltransferase (TdT) inhibitors from natural resources, such as the fermentation products of *Actinomycetes* and fungi and the extracts of many species of higher plants and animals. For this purpose, more than 20,000 species of *Actinomycetes*, fungi, higher plants and animals were used. However, we could not find an inhibitor that was completely specific for TdT although several compounds showed selective inhibition of the polymerase. One of the latter was a natural compound from the Japanese vegetable, *Arctium lappa*, that was a potent and selective inhibitor of TdT. *Arctium lappa* is an edible vegetable with the common name of gobo and is cultivated and eaten only in Japan. A water-soluble extract from the plant was found to have a potent inhibitory effect on the activity of human TdT, but to have only a weak effect on mammalian α, β, δ and λ DNA polymerases.

We then set out to purify the inhibitor from the gobo extract and to determine its chemical structure. Roots of *Arctium lappa* (1 kg dry wt) were sliced and extracted in 60% methanol for 1 day. After filtration, the 60% methanol extract was evaporated leaving a dark brown and waxy material that was then redissolved in methanol. The extract was partitioned between ethyl acetate (1 l) and water (1 l). The water layer extract was in turn partitioned between n-butanol (1 l) and water (1 l). We found that the n-butanol layer extract inhibited the activity of TdT. The n-butanol