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Structure–activity relationship of coumarin derivatives on xanthine oxidase-inhibiting and free radical-scavenging activities

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ABSTRACT

We employed 1,1-diphenyl-2-picrylhydrazyl hydrate (DPPH)– and 5,5-dimethyl-1-pyrroline-N-oxide (DMPO)–electron spin resonance (ESR) to study the effects of suppression of reactive oxygen species (ROS) by eight selected coumarin derivatives under oxidative conditions. Esculetin was the most potent radical scavenger among the eight tested compounds. Our results suggest that the number of hydroxyl groups on the ring structure of coumarins is correlated with the effects of ROS suppression. We also investigated the effect of the derivatives on the inhibition of xanthine oxidase (XO) activity, and the structure–activity relationships (SARs) of these derivatives against XO activity were further examined using computer-aided molecular modeling. All determined derivatives competitively inhibited XO. The results of the structure-based molecular modeling exhibited interactions between coumarins and the molybdopterin region of XO. The carbonyl pointed toward the Arg880, and the ester O atom formed hydrogen bonds with Thr1010. Esculetin, which bears two hydroxyl moieties on its benzene rings, had the highest affinity toward the binding site of XO, and this was mainly due to the interaction of 6-hydroxyl with the E802 residue of XO. The hypoxanthine/XO reaction in the DMPO-ESR technique was used to assess the combined effect on enzyme inhibition and ROS suppression by these coumarins, and the results showed that esculetin was the most potent agent among the tested compounds. We further evaluated the effects of the test compounds on living cells, and esculetin was still the most potent agent at protecting cells against ROS-mediated Aβ-damage among the tested coumarins.

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1. Introduction

A redox imbalance in a healthy living system leads to malfunctioning of cells that ultimately results in various diseases, including cancer, neurological degeneration, and arthritis as well as accelerating the aging process. These consequences become even more harmful when genetic variations impair the normal degradation of these altered proteins. Therefore, therapeutic strategies should aim at reducing free-radical formation and scavenging free radicals [1]. The toxicity ascribed to the superoxide radical is believed to be caused by superoxide's direct interaction with biological targets. Reactive oxygen species (ROS) can initiate a wide range of toxic oxidative reactions [2]. ROS released by phagocytic cells are involved in the link between inflammation and cancer. Excessive and persistent formation of ROS by inflammatory cells is thought to be a key factor in their genotoxic effects. Intracellular ROS production is associated with a number of cellular events including activation of NAD(P)H oxidase, xanthine oxidase (XO), and the cellular mitochondrial respiratory chain [3].

XO is an important source of free radicals and has been reported in various physiological and pathological models. XO causes gout and is responsible for oxidative damage to living tissues. This enzyme reduces molecular oxygen, leading to the formation of $O_2^{\bullet-}$ and hydrogen peroxide. Regulation of XO activity is important during inflammation [4]. Treatment with an XO inhibitor largely prevented the development of endothelial dysfunction and atherosclerosis in mice [5]. XO catalyses the oxidation of hypoxanthine and xanthine to uric acid yielding superoxide radicals and raises the oxidative level in an organism. Hydroxylation takes place at the molybdopterin center (Mo-pt) via an Mo–OH oxygen which forms a bond with a carbon atom of the substrate such that the oxygen atom is derived from water rather than molecular oxygen [6]. The active form of XO is as a homodimer with a molecular weight of 290 kDa, with each of the monomers acting independently during catalysis. Each subunit contains one molybdopterin cofactor, two distinct [2Fe–2S] centers, and one FAD cofactor. The co-crystal structure of salicylate–XO was first reported by Enroth et al. [7] and was provided for structure-based docking studies.

Natural polyphenols can be divided into several different classes depending on their basic chemical structure which ranges from simple molecules to highly polymerized compounds. Coumarin (known as 1,2-benzopyrone), consisting of fused benzene and α-pyrone rings, is an important group of low-molecular weight phenolics [8] and has been widely used for the prevention and treatment of venous thromboembolism, myocardial infarction and strokes [9]. Coumarins acting to inhibit XO inhibition have been reported [10]. The structure–activity relationships (SARs) of coumarins interacting with this enzyme have also been discussed [11,12]. However, the influences of enzyme–substrate binding by coumarins and the stereochemistry on XO have not been characterized. In this study, the protective effect of some coumarins against ROS and their influence on binding to the active site of XO by various substitution groups and positions on coumarins were investigated. We also combined the ROS-scavenging and XO-inhibition roles of coumarins in order to identify which compounds are more vital to therapeutic applications. We eventually applied our results to living cells to confirm the conclusions drawn from the *in vitro* experiments.

2. Materials and methods

2.1. Materials

Coumarin, 4-hydroxycoumarin, 7-hydroxycoumarin, esculetin, scopoletin, dihydrocoumarin, 4-methylesculetin, and 7-hydroxy-4-methylcoumarin were purchased from ACROS (Geel, Belgium) (Fig. 1). XO (XO, EC 1.2.3.2.), xanthine, and allopurinol were purchased from Sigma (St. Louis, MO). All of the solvents used in this study were from E. Merck (Darmstadt, Germany). Minimum essential medium (MEM), fetal bovine serum (FBS), penicillin, and streptomycin were obtained from Gibco BRL (Grand Island, NY). β-Amyloid peptide ($A\beta_{25-35}$ fragment) was purchased from Jerini Peptide Technologies (Berlin, Germany).

2.2. Cell culture

Neuro-2A neuroblastoma cells (BCRC 60026) were purchased from CCRC (Culture Collection and Research Center, Hsinchu, Taiwan). Cells were grown in MEM containing 10% FBS, 1% nonessential amino acid, and 100 μg/ml penicillin–streptomycin. Conditions were maintained in humidified 95% air/5% CO_2 incubator at 37 °C.

2.3. DPPH radical-scavenging assay

The reaction was performed in 3 ml of methanol containing 250 μM of freshly prepared 1,1-diphenyl-2-picrylhydrazyl hydrate (DPPH). The reaction mixtures were protected from light and incubated for 90 min at room temperature, after which the absorbance of the remaining DPPH was determined colorimetrically at 517 nm. The scavenging activities of coumarins (100 μM) were measured as the decrease in absorbance of DPPH expressed as a percentage of the absorbance of a control DPPH solution without coumarins [13].

2.4. XO activity assay

The enzyme activity was measured spectrophotometrically by continuously measuring uric acid formation at 295 nm with xanthine as the substrate. The XO assay consisted of a 500-μl reaction mixture containing 7.5 mM phosphate buffer, 20 mM 3-(cyclohexylamino)-1-propanesulfonic acid (CAPS), 38 μM EDTA (pH 7.0), 3 U/l XO, and 50 μM xanthine as the substrate. The assay was initiated by adding the enzyme to the reaction mixture without or with inhibitors. The assay mixture was incubated for 3 min at 37 °C, and absorbency readings were taken every 5 s [14]. The extent of inhibition was expressed as the chemical concentration required to inhibit 50% of the enzyme activity (IC_{50}). The inhibition type was determined by the Lineweaver–Burk plot. The substrate concentrations were 20, 40, and 60 μM, respectively, in the reaction mixture without or with inhibitors. All data obtained from the enzyme assays

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