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Notes & Tips

Microplate screening assay to identify inhibitors of human catechol-*O*-methyltransferase

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O-methylation by catechol-*O*-methyltransferase (COMT)[1] is an important metabolic pathway leading to inactivation of catecholamine neurotransmitters such as norepinephrine and dopamine [1] and elimination of catechol steroids and xenobiotic catechols [2]. Levodopa remains the most effective drug for treatment of Parkinson's disease [1]. To enhance its availability in the brain, where it is decarboxylated, resulting in release of dopamine, it is usually given with a dopa-decarboxylase inhibitor, which blocks its peripheral breakdown. Under these conditions, levodopa is metabolized in the periphery via an alternative path involving COMT, which degrades it to 3-*O*-methyldopa. If COMT could be blocked in the periphery, more levodopa would therefore be available in the brain. Two COMT inhibitors, entacapone, which acts mainly in the periphery, and tolcapone, which acts both centrally and peripherally, have been developed. Clinical use of tolcapone has, however, been restricted, because there have been reports of liver toxicity [3, and refs. within]. Although there has been a recent report concerning one potent COMT inhibitor [4], more are needed. A rapid, robust, and sensitive means of determining COMT activity would be valuable in relation to the development of COMT inhibitors.

Several methods of measuring COMT activity have been developed [5–7], but none is well suited to automation and high throughputs. In this report we describe development of a 96-well microplate assay to identify inhibitors of human S-COMT, using aesculetin as substrate. Methylation of aesculetin to scopoletin is measured directly in a reaction mixture in which *S*-(5′-adenosyl)-L-methionine (AdoMet) serves as methyl-group donor. The reaction is followed fluorometrically.

The human gene segment encoding the water-soluble form of COMT, S-COMT, was amplified from total brain RNA (Invitrogen) and subcloned into the pBAD/Myc-HisC plasmid (Invitrogen). Expression of recombinant S-COMT in *Escherichia coli* cells was induced by arabinose. The cells were collected by centrifugation (10 min, 3000*g*, 4 °C). The cells (the centrifugation pellet) were suspended in 1 ml of buffer solution (50 mM phosphate, 300 mM NaCl, 5 mM $MgCl_2$, pH 7.4) and disrupted by treatment with lysozyme and DNase I followed by sonication. The supernatant from the subsequent centrifugation (the cytosolic fraction) was used as an S-COMT source in the studies. It was stored at −20 °C until use. Aesculetin was obtained from Extrasynthèse (d502); catechol (C-9510), 4-chlorocatechol (Library of Rare Chemicals), 4-nitrocatechol (N-7126), and 3,5-dinitrocatechol (D-131) were bought from Sigma; and 3,4-dihydroxybenzoic acid (D 10,980-0) and 2,3-dihydroxynaphthalene (D 11,600-9) were bought from Aldrich. The aesculetin and competing catechol substrates were dissolved in dimethyl sulfoxide (DMSO) and diluted with aqueous buffer solution (100 mM phosphate, 5 mM $MgCl_2$, 20 mM L-cysteine, pH 7.4) for a final DMSO concentration of 2% in the 100 µl of reaction mixture. All of the reagents were dissolved in the same buffer solution, since the presence of magnesium ions is essential for COMT-catalyzed methylation, and purified human S-COMT requires cysteine as a reducing agent to maintain its activity [8]. For each sample, inhibitor and aesculetin solutions were placed in 3 wells of a

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[1] *Abbrevations used:* COMT, catechol-*O*-methyltransferase; DMSO, dimethyl sulfoxide.

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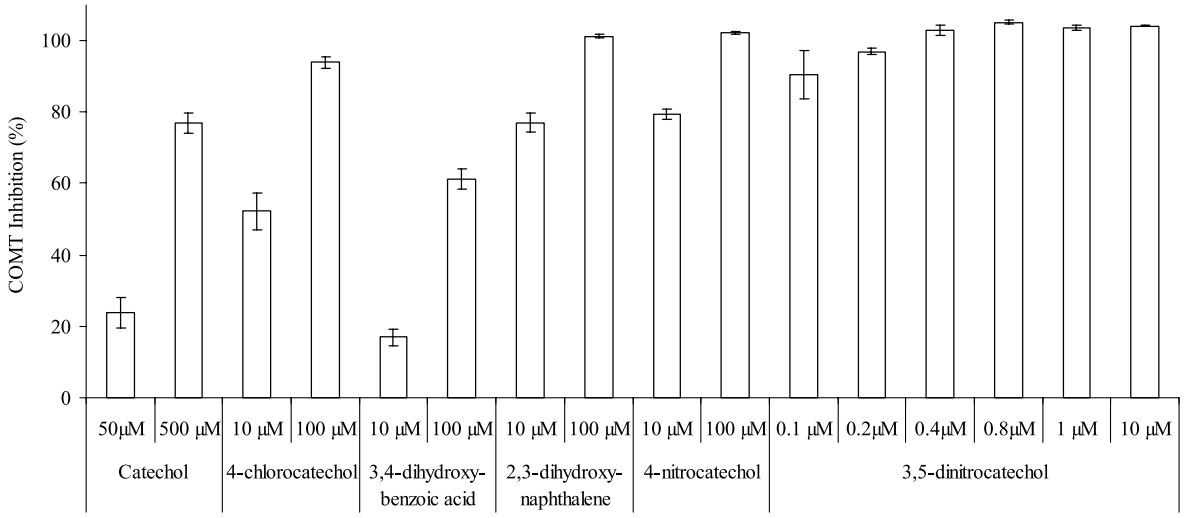

Fig. 1. Activities of inhibitors measured using a new, homogeneous 96-well assay for primary screening of S-COMT inhibition. The positive control used was 3,5-dinitrocatechol. Measurements were made at several concentrations.

standard flat-bottomed 96-well plate (Nunc Micro Well Plate; Cat. No. 442587). Controls without inhibitor or AdoMet were included in each microplate. Positions on the plate were varied to eliminate systematic error. The plate was placed on ice, and the cytosolic fraction containing the enzyme was added to a final protein concentration of 15 μg/ml. A preincubation period of 5 min was started by placing the plate in the microplate reader set at 37 °C. The reaction was initiated by addition of AdoMet, at 37 °C, for a final concentration of 10 μM. The reaction was followed for 60 min using a Wallac Victor^2 1420 multilabel microplate counter (Perkin–Elmer Life and Analytical Sciences). Fluorescence was measured at 4-min intervals. The excitation and emission wavelengths were 355 nm (40-nm bandwith) and 460 nm (25-nm bandwith), respectively. The change in fluorescence caused by enzymatic *O*-methylation of aesculetin to scopoletin was used to describe inhibitory activity. The activity was estimated in each reaction mixture from the fluorescence at 60 min, after subtraction of fluorescence at the beginning of the assay. Two concentrations of each inhibitor were tested (Fig. 1), in a range in which they have previously been reported to be active [2]. A known tight-binding inhibitor of COMT, 3,5-dinitrocatechol [9], was used as a positive control, at several concentrations. Results are shown in Fig. 1. The dose–response curves for 3,5-dinitrocatechol (Fig. 2) were obtained using the method described here and an HPLC method described previously [2], after termination of reaction by addition of 10 μl of cold 4 M perchloric acid. Use of a reference standard (scopoletin, S-2500; Sigma) showed that 30% of aesculetin was methylated in the inhibitor-free reaction mixture. The IC_{50} for 3,5-dinitrocatechol under these conditions was approximately 35 nM, similar to the previously reported value of 12 nM [9].

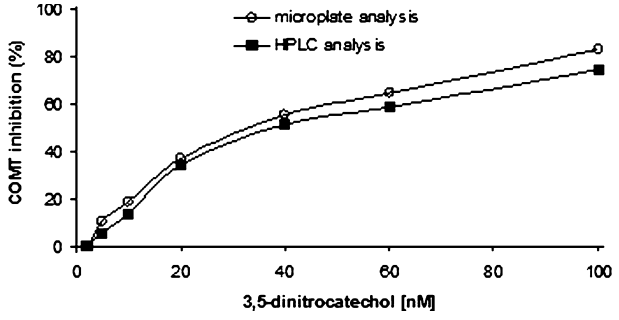

Fig. 2. Dose–response curves for 3,5-dinitrocatechol. HPLC analysis was undertaken using the samples employed in microplate analysis, after termination of the reactions by the addition of perchloric acid. COMT inhibition (%) was estimated in relation to the inhibitor-free reaction mixture. Activities in microplate analysis were calculated by subtracting fluorescence values at the beginning of the assay from those after 60 min. Scopoletin was separated during HPLC analysis and quantified using an authentic reference standard.

The quality of the 96-well plate assay was assessed by calculating signal-to-noise (S/N) ratios as described [10] and signal-to-background (S/B) ratios and Z′ factors as described [11]. The mean S/B ratio ± SD ($n = 16$) in these experiments was 3.0 ± 0.4. The mean S/N ratio ± SD and mean Z′ factor ± SD were 18.5 ± 12.9 and 0.68 ± 0.21, respectively. On the basis of the values obtained, we decided that a S/B ratio $\geqslant 2.6$, a S/N ratio $\geqslant 10$, and a Z′ factor $\geqslant 0.5$ should be regarded as acceptance criteria in routine experiments. Inhibition with the positive control, 3,5-dinitrocatechol, at a concentration of 0.1 μM, was 90.4 ± 6.7% (mean ± SE, $n = 3$). Acceptance limits for the positive control were set at 85–95% inhibition. Plate-to-plate and day-to-day variations in relation to the positive control results were 1.0 and 12.8%, respectively. We believe that these values indicate good reliability and reproducibility.

The major advantages of the new assay are that it is homogeneous and that throughputs for primary screening of S-COMT inhibition can be high. The method should find extensive use in screening small-molecule inhibitors of human S-COMT, because it can easily be automated. Preliminary results indicate that the method also works well when 384-well microplates are employed.

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