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Specific plasma membrane binding sites for polyphenols, including resveratrol, in the rat brain

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Abbreviations: **:** aryl hydrocarbon receptor; AhR; beta amyloid, Aβ; epidermal growth factor, EGF; (-)-Epicatechin, EC; (-)-Epicatechin gallate, ECG; (-)-Epigallocatechin, EGC; (-)-Epigallocatechin gallate, EGCG; protein kinase C, PKC; insulin-like growth factor, IGF.

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ABSTRACT

Using [^{3}H]-resveratrol as radioligand, we investigated the possible existence of specific polyphenol binding sites at the level of the cellular plasma membrane in rat brain. Specific [^{3}H]-resveratrol binding sites were found to be enriched in the plasma membrane pellet with lower levels in the nuclear and cell debris fraction. Specific [^{3}H]-resveratrol binding to the plasma membrane fraction was sensitive to trypsin digestion and protein denaturation but not to DNAse and RNAse treatment. Saturation binding experiments revealed that specific [^{3}H]-resveratrol recognized a single class of sites with an apparent affinity (K_D) of 220 ± 45 nM and a maximal capacity (Bmax) of 1060 ± 120 fmol/mg protein. Various polyphenols and resveratrol derivatives competed against specific [^{3}H]-resveratrol binding in rat brain plasma membrane homogenates with the tea catechins gallates (epigallocatechin gallate and epicatechin gallate) displaying the highest affinities (Ki = 25-45 nM) followed by resveratrol (Ki = 102 nM). Quantitative autoradiographic studies revealed that specific [^{3}H]-resveratrol binding sites are broadly distributed in the rat brain, with highest levels of labeling seen in the choroid plexus and subfornical organ. Finally, the potency of various polyphenols and resveratrol analogues in protecting hippocampal cells against β-amyloid-induced toxicity correlated well (r=0.74) with their apparent affinity in the [^{3}H]-resveratrol binding assay. Taken together, these results suggest that the neuroprotective action of various polyphenols and resveratrol analogues could be mediated by the activation of common “receptor” binding sites particularly enriched at the level of the cellular plasma membrane in the rat brain.

INTRODUCTION

Polyphenols are secondary plant metabolites found in foods and beverages that contain one or more phenolic hydroxyl groups attached to at least one carbon-based aromatic ring (Fig. 1). Epidemiological and animal studies suggest that polyphenols including resveratrol (3,5,4'-trihydroxy-trans-stilbene), a naturally occurring stilbene found in red wine, and tea catechins exhibit pleiotropic activities such as anti-inflammatory and anti-carcinogenic activities as well as having beneficial effects against cardiovascular diseases (Belleville, 2002; Gescher, 2004). Moreover, there is a growing number of studies indicating that natural polyphenol extracts may reduce the incidence of age-related neurological disorders including macular degeneration, stroke and dementia (Orgogozo et al., 1997; Commenges et al., 2000; Bastianetto and Quirion, 2002). It has thus been postulated that regular consumption of various beverages (e.g. tea, red wine), vegetables and fruits that contains high amounts of polyphenolic compounds are likely to contribute to health (for review see (Arts and Hollman, 2005).

In vitro studies using peripheral cell lines (e.g. endothelial and breast cancer cells) suggest that the purported effects of polyphenols (e.g. tea catechins, resveratrol and piceatannol) may be attributable to their interaction with the enzyme cyclooxygenase (COX) especially COX-2 (Murias et al., 2004), heme oxygenase-1 (Dore, 2005), sirtuins (Kaeberlein et al., 2005), androgen and/or estrogen receptors (Bowers et al., 2000; Gao et al., 2004), insulin-like growth factor-1 (IGF-1) or epidermal growth factor (EGF) receptors (Shimizu et al., 2005; Hou et al., 2005), aryl hydrocarbon receptors (AhR) (Casper et al., 1999; Palermo et al., 2003) and the 67 kDa laminin receptors (67 kDa LR)

(Tachibana et al., 2004). Accordingly, these polyphenols are able to target a variety of cellular sites to induce their effects.

More recently, *in vitro* and a limited number of *in vivo* studies have demonstrated that resveratrol and flavonoids (e.g. tea catechins) exert neuroprotective effects in various models of toxicity (Bastianetto and Quirion, 2002; Esposito et al., 2002; Dore, 2005; Simonyi et al., 2005; Bastianetto et al., 2006), suggesting that they could contribute to the beneficial effects of foods and beverages in reducing the risk of developing neurological disorders (Arts and Hollman, 2005). It has been postulated that the neuroprotective effects of polyphenols are not solely due to their antioxidant activities but likely involve modulatory effects on signal transduction pathways (Esposito et al., 2002; Han et al., 2004). For example, resveratrol was shown to be able to protect hippocampal cells against beta-amyloid (Aß)-induced toxicity through its ability to rapidly activate protein kinase C (Han et al., 2004) while another study reported that resveratrol markedly reduced NF-kappaB signaling stimulated by $Aß_{1-42}$ (Chen et al., 2005). Some of the modulatory effects of resveratrol on intracellular effectors are shared by green tea-derived constituents such as epigallocatechin gallate (EGCG) with a role for the PKC pathway (Levites et al., 2003).

On the basis of these observations, we postulated that resveratrol and related polyphenols could regulate, at least partly, neuronal cell functions through specific plasma membrane "receptor"-like mechanism. Accordingly, the main objective of the present study was to identify and characterize the presence of specific binding sites for [^{3}H]-resveratrol in the

rat brain. Our results reveal the presence of specific [^{3}H]-resveratrol binding sites enriched in the plasma membrane fraction of the rat brain. These binding sites are discretely distributed and competed by various polyphenols and catechin gallate esters with affinities relevant to their neuroprotective abilities against Aß-induced neurotoxicity.

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MATERIALS & METHODS

Animals and reagents

Male Sprague-Dawley rats (250-300 g) were obtained from Charles River (St-Constant, QC). Resveratrol (3,4',5-trihydroxy-trans-stilbene), piceatannol (3,3',4',5-tetrahydroxy-trans-stilbene), trans-stilbene (trans-1,2-diphenylethylene), benzo[a]pyrene, estradiol, diethylstilbestrol and epicatechin derivatives were purchased from Sigma-Aldrich (St. Louis, MO, USA). Resveratrol derivatives (Fig. 1) including 4,4'-dihydroxy-trans-stilbene, 4-hydroxy-4'-methoxy-trans-stilbene, 4-hydroxy-trans-stilbene, 3,5-dihydroxy-4'-methoxy-trans-stilbene, 3,5-dimethoxy-4'-hydroxy-trans-stilbene, 3,4,4'-trihydroxy-trans-stilbene, 3,4'-dihydroxy-4-methoxy-trans-stilbene, and 4,4'-dihydroxy-3-methoxy-trans-stilbene were kindly provided by Prof. Luca Forti (Dipartimento di Chimica, Universita di Modena e Reggio Emilia, Italy), whereas 3,4-dihydroxy-trans-stilbene and 3,4,5-trihydroxy-trans-stilbene were kindly provided by Prof. Zhong-Li Liu (National Laboratory of Applied Organic Chemistry, Lanzhou University, China) and 3,5-dihydroxy-trans-stilbene was kindly provided by Prof. Yoshihisa Takaishi, Faculty of Pharmaceutical Sciences, University of Tokushima, Japan. The fragment 25-35 of amyloid beta ($A\beta_{25-35}$) was kindly provided by P. Gaudreau (CHUM, University of Montreal, Montreal, Quebec, Canada). [^{3}H]-resveratrol was purchased from Moravek Biochemicals Inc. (Brea, CA, USA). Materials used for cell cultures were obtained from Invitrogen (Burlington, Ontario, Canada) while other products were from Sigma-Aldrich or Fisher scientific (Montreal, QC, Canada). Animal care was according to protocols and guidelines of the McGill University Animal Care Committee in accordance with regulations of the Canadian Council for Animal Care.

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Membrane Preparations

Membranes were prepared from rat brains as previously described (Dumont et al., 1998), with minor modifications. Briefly, rats were decapitated and their brains were rapidly removed and homogenized in a Krebs ringer phosphate (KRP) buffer, pH 7.4, containing NaCl (135 mM), KCl (3.6 mM), NaH_2PO_4 (5 mM), $MgCl_2$ (0.5 mM), $CaCl_2$ (1.5 mM) and HEPES (10 mM), using a Brinkman polytron (at setting 6 for 15-20 sec). Homogenates were centrifuged at 2,000×g for 15 min, the pellets were re-homogenized in the above buffer and centrifuged again at 2,000×g. The resulting pellets were used as PI, mainly containing nucleus and cell debris; the supernatants were pooled and centrifuged at 49,000×g for 20 min. These pellets were used as PII as partially purified plasma membrane preparations. The supernatants were used as SII, mainly containing soluble proteins.

[^{3}H]-resveratrol binding assay

All binding assays were initiated by adding 100 µl of membrane preparations in a final volume of 500 µl of Krebs buffer containing 0.1% (w/v) BSA, 0.05% (w/v) bacitracin, [^{3}H]-resveratrol and competitor as indicated. Isotherm saturations and competition binding assays were performed at room temperature. Saturation experiments were performed in the presence of increasing concentrations of [^{3}H]-resveratrol whereas competition binding experiments were performed in the presence of 20 nM [^{3}H]-resveratrol and various competitors at concentrations ranging from 10^{-10} to 10^{-4} M. Non-specific binding was determined in the presence of 100 µM resveratrol. After a 1 hr

incubation, the binding reaction was terminated by rapid filtration through Schleicher and Schuell #32 glass filters (previously soaked in 1.0% polyethyleneimine) using a cell harvester filtering apparatus (Brandel Instruments, Gaithersburg, MD, USA). Filters were rinsed three times with 3 ml cold KRB buffer and radioactivity remaining on filters was quantified using a beta counter with 45% efficiency (Beckman Instruments, Meriden CT, USA).

Quantitative receptor autoradiography

Receptor autoradiography was performed as described in details elsewhere (Dumont et al., 1998). Briefly, rats were sacrificed by decapitation, and their brains were rapidly removed from the skull, frozen in 2-methylbutane at -40^{o}C for 15 sec, and then kept at -80^{o}C until needed. Sections (20 μm) were obtained using a cryomicrotome at -17^{o}C, mounted on gelatin-chrome-alum-coated slides, dried overnight in a desiccator at 4^{o}C, and then kept at -80^{o}C until use.

Adjacent coronal sections were pre-incubated for 60 min at room temperature in a KRB buffer at pH 7.4 and then incubated for 60 min in a fresh preparation of KRB buffer containing 20 nM [^{3}H]-resveratrol in the presence or absence of 100 μM resveratrol. Following a 60 min incubation, sections were washed four times, 1 min each in ice-cold KRB buffer then dipped in deionized water to remove salts and rapidly dried. Incubated sections were apposed against Kodak Biomax MR films (PerkinElmer, Woodbridge, ON, Canada), for three months alongside with ^{3}H radioactive standards (GE Healthcare Canada, Baie d'Urfe, QC, Canada).

Primary hippocampal cell cultures

Hippocampal cell cultures were prepared from E19-E20 fetuses obtained from Sprague-Dawley rats as described before with minor modifications (Bastianetto et al., 2000a). Cells were grown at day 0 (density of approximately 4 x 10^4 viable cells per well in 96-well plates) in HEPES-buffered D-MEM high glucose medium supplemented with KCl (20 mM) and fetal bovine serum 10% (v/v). The initial medium was removed at days 1 and 5 and replaced with the same medium containing N2 supplement 1% (v/v). The experiments were performed using 6-day-old cultures, at which time the hippocampal neurons are fully differentiated (Mattson et al., 1991).

Aß-induced toxicity and assessment of cell viability

On the day of experiment, the medium was removed and cells were incubated at 37ºC in the same medium without N2 supplement and exposed for 24 hours to fresh $Aß_{25-35}$ (25 μM) in the presence or absence of different compounds. Cell viability was evaluated 24 hours later using the MTT colorimetric assay, as described earlier (Bastianetto et al., 2000a). Optical density (OD) was determined at 570 nm using a micro-plate reader (Bio-Tek Instruments® Inc., Montreal, QC, Canada).

Statistical analysis

All binding experiments were repeated at least three times (each in triplicate) and results (mean ± S.E.M.) expressed as percentage of specific binding or in fmol/mg of protein. Binding parameters were determined using GraphPad Prism program (version 3.03)

(GraphPad Software Inc. San Diego, CA). The affinity (K_D) and maximal binding capacity (Bmax) of the saturation isotherms were estimated by a nonlinear regression curves and were fitted to a one-site and two-site model using hyperbola equations with $p < 0.05$ considered significant using the Fisher test. Saturation isotherm curves were best fitted to a one site model ($p > 0.05$). The concentration of competitor required to compete for 50% of specific [^{3}H]-resveratrol binding was calculated from competition binding curves and results expressed as percentage of specific binding representing the mean ± S.E.M. of three to four individual determinations, each in triplicate. Competition curves were fitted to a one-site and a two-site models using GraphPad Prism program (version 3.03) with $p < 0.05$ considered significant using the Fisher test. Competition binding curves were best fitted to a one-site model ($p > 0.05$). Ki values were determined according to the equation of Cheng and Prusoff ($Ki = IC_{50}/(1 + C/K_D)$) (Cheng and Prusoff, 1973). The optical density of the autoradiographic images was measured from Kodak MR films using a microcomputer-assisted video imaging densitometer (MCID system, Imaging Research, Ste-Catharines, ON, Canada). Survival of vehicle-treated control groups not exposed to $Aß_{25-35}$ or drugs was defined as 100%, and the number of surviving cells in the treated groups was expressed as percent of controls. One-way ANOVA followed by a Newman Keuls' multiple comparison test was used to compare control and treated groups with $p < 0.05$ being considered significant. Cell cultures experiments were repeated four times (each in sextuplicates). EC_{50} values (concentration of compounds required to block 50% of cell death induced by $Aß_{25-35}$ alone) were calculated using GraphPad Prism.

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RESULTS

To determine the cellular fraction(s) enriched in polyphenol binding sites, the binding of [^{3}H]-resveratrol was evaluated in rat brain subcellular fractions using 20 nM of radioligand. As shown in Table 1, significant [^{3}H]-resveratrol binding was detected in nuclear and large cellular components (PI pellet) as well as in plasma membrane (PII pellet), while soluble proteins (SII fraction) contained much lower levels of specific binding. In fact, the plasma membrane fraction (PII pellet) is the most enriched one in specific [^{3}H]-resveratrol binding. Moreover, [^{3}H]-resveratrol binding to the PII fraction is significantly reduced by pre-treatment with trypsin (0.25% at 37^{o}C for 10 min) or boiling cellular fractions for 10 min, but not by pre-treatment with benzonase (50 U/ml for 10 min at room temperature), a mixture of DNase and RNase (Table 1). These results suggest that specific [^{3}H]-resveratrol binding sites are of proteinous nature and particularly enriched in the plasma membrane. Accordingly, The PII fraction was used for subsequent binding experiments.

Saturation binding parameters of specific [^{3}H]-resveratrol binding were investigated next (Fig. 2). Scatchard transformation of the isotherm saturation binding experiments suggests that [^{3}H]-resveratrol specifically binds to an apparent single class of sites (Figure 2, insert). Curve fitting analysis revealed that [^{3}H]-resveratrol specifically binds to a saturable class of sites (Bmax 1060 fmol/mg protein) with an apparent affinity (K_D) of 220 ± 80 nM, in rat brain membrane homogenates (PII fraction; Fig. 2).

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A series of compounds with structural similarities to resveratrol and tea catechins (Fig. 1) were evaluated next for their ability to compete for specific [^{3}H] resveratrol binding in rat brain membrane homogenates (PII fraction). As shown in Figure 3, (-)-epigallocatechin gallate (EGCG) and (-)-epicatechin gallate (ECG) are most potent to compete for specific [^{3}H]-resveratrol binding with Ki value of 25 and 45 nM, respectively, while resveratrol was found to be two to four times less potent (Ki = 102 nM) (Table 2). Among the structurally related analogues of resveratrol tested here, 3,5-dihydroxy-trans-stilbene, piceatannol, 3,4,4'-trihydroxy-trans-stilbene and 3,5-dimethoxy-4'-hydroxy-trans-stilbene were the only compounds that were able to significantly compete against specific [^{3}H]-resveratrol binding (Fig. 3 and Table 2). Inactive molecules (up to 10 μM) include estradiol, benzo[a]pyrene, (-)epicatechin, (-)-epigallocatechin, trans-stilbene, *trans*-4'-stilbenemethanol, diethylstilbestrol, 3,4-dihydroxystilbene, 3,4,5-trihydroxystilbene, 4,4'-dihydroxy-trans-stilbene, 4-hydroxy-4'-methoxy-trans-stilbene, 3,5-dihydroxy-4'-methoxy-trans-stilbene, 3,4'-dihydroxy-4-methoxy-trans-stilbene and 4,4'-dihydroxy-3-methoxy-trans-stilbene (Table 2).

We evaluated next the discrete distribution of specific [^{3}H]-resveratrol binding sites in rat brain using quantitative autoradiography. As shown in Figure 4A, [^{3}H]-resveratrol binding is widely distributed in the rat brain, with highest levels seen in the choroid plexus and subfornical organ, and lower but significant amounts found in other regions such as the hippocampal formation and the cortex (Fig. 4A). A quantitative analysis confirmed the broad distribution of specific [^{3}H]-resveratrol binding in the rat brain (Fig. 4B).

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A functional assay was then used to test the capacity of resveratrol, homologues and catechins to protect hippocampal cells against $A\beta_{25-35}$-induced toxicity. This assay was chosen because of the purported protective effects of resveratrol and tea-derived catechins in these cell types that were exposed to either $A\beta_{25-35,}$ $A\beta_{1-40}$ or $A\beta_{1-42}$ (Han et al., 2004; Bastianetto et al., 2006). The catechin gallates EGCG (EC_{50} = 5 μM) and ECG (EC_{50} = 7 μM) and the resveratrol analog, 3,4,4'-trihydroxy-trans-stilbene (EC_{50} = 6 μM), were the most potent neuroprotective agents (Table 2). Additionally, piceatannol (EC_{50} = 11 μM), resveratrol (EC_{50} = 13 μM), 3,5-dihydroxy-trans-stilbene (EC_{50} = 17 μM) and 3,5-dimethoxy-4'-hydroxy-trans-stilbene (EC_{50} = 26 μM) also exerted neuroprotective effects, albeit with lower potencies (Table 2). In contrast, estradiol, benzo[a]pyrene, trans-stilbene, trans-4'-stilbenemethanol, diethylstilbestrol, 3,4-dihydroxystilbene, 3,4,5-trihydroxystilbene, epicatechin, epigallocatechin, 4,4'-dihydroxy-trans-stilbene, 4-hydroxy-4'-methoxy-trans-stilbene, 3,5-dihydroxy-4'-methoxy-trans-stilben, 3,4'-dihydroxy-4'-methoxy-trans-stilbene and 4,4'-dihydroxy-3-methoxy-trans-stilbene were ineffective at up to 50 μM (Table 2) or even neurotoxic by themselves (data not shown). Interestingly, the affinity of polyphenols and various resveratrol analogs to compete for specific [^{3}H]-resveratrol binding correlated very well (r = 0.74) with their neuroprotective activity against $A\beta_{25-35}$-induced toxicity in primary hippocampal cell culture (Fig. 5), suggesting that the neuroprotective effect exerted by these phenolic compounds could be mediated by a common mechanism involving a specific plasma membrane protein.

DISCUSSION

In this study, we demonstrated the existence of specific cell plasma membrane binding sites for polyphenols in the rat brain. Saturation binding studies revealed that [^{3}H]-resveratrol binds to saturable amounts of a single population of sites with an apparent affinity of 220 nM and a maximal capacity of 1060 fmol/mg protein in rat brain plasma membrane homogenates. Competition binding experiments using various analogues of resveratrol and other phenolic compounds demonstrated that tea catechin gallates followed by resveratrol are the most potent to compete for specific [^{3}H]-resveratrol binding sites. Other structural analogues including 3,5-dihydroxy-trans-stilbene, piceatannol, 3,4,4'-trihydroxy-trans-stilbene and 3,5-dimethoxy-4'-hydroxy-trans-stilbene were also able to compete for specific [^{3}H]-resveratrol binding, but with lower affinities. Interestingly, structure-activity studies revealed that these molecules are also able to protect hippocampal cells against toxicity induced by $A\beta_{25-35.}$ In contrast, estradiol, trans-stilbene, diethylstilbestrol, benzo[a]pyrene and the catechin non-gallate esters (i.e. epicatechin and epigallocatechin), which failed to compete for specific [^{3}H]-resveratrol binding at concentrations up to 10 μM, failed to protect cells against $A\beta_{25-35}$-induced toxicity. Taken together, these results suggest the existence, at the level of the cell plasma membrane, of specific resveratrol receptor binding sites that have functional relevance for the neuroprotective effects of these molecules.

Resveratrol and tea catechins possess a wide range of biological and pharmacological properties (see Introduction). For example, it has been shown that polyphenolic compounds contained in grapes and teas have potent protective effects in various models

of neurotoxicity (Bastianetto et al., 2000b; Levites et al., 2003; Lee et al., 2004; Han et al., 2004; Bastianetto et al., 2006). These results are in agreement with the neuroprotective effects of the tea catechin gallates, EGCG and ECG, and resveratrol reported in the present study. However, limited information is currently available on the first biochemical or molecular step leading to these events. The recent series of findings demonstrating that polyphenols and tea catechins can regulate various intracellular pathways such as mitogen-activated protein kinases (MAPK) (Schroeter et al., 2002), protein kinase C (PKC) (Levites et al., 2003; Han et al., 2004), pro-apoptotic proteins (e.g. bax and Bcl-XL) (Choi et al., 2001), caspases (Katunuma et al., 2004), sirtuins (Kaeberlein et al., 2005) and heme oxygenase-1 (Dore, 2005) may be taken as an indication for the existence of an upstream site of action at the level of the plasma membrane. This hypothesis is indeed supported by our findings that the highest level of specific [^{3}H]-resveratrol binding was detected in the plasma membrane-enriched fractions (PII). Moreover, plasma membrane specific [^{3}H]-resveratrol binding was markedly reduced by treatments with trypsin and protein denaturation (boiling), but not by benzonase treatment, suggesting its proteinaceous nature. Saturation binding experiments revealed that [^{3}H]-resveratrol bound to saturable amounts of specific sites with an apparent affinity of 220 nM in the rat brain. Additionally, competition binding experiments demonstrated that specific [^{3}H]-resveratrol binding was competed by various natural and synthetic polyphenolic compounds with EGCG, ECG and resveratrol being most potent. Interestingly, the apparent affinity of these molecules to compete for specific [^{3}H]-resveratrol binding correlated well ($r = 0.74$) with their abilities to protect hippocampal neurons against Aβ-induced neurotoxicity. These structure-activity data

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strongly suggest that specific [^{3}H]-resveratrol binding sites present at the level of the plasma membrane are functionally relevant and may serve as polyphenol "receptors" initiating a cascade of events leading to their various biological effects.

Earlier studies have shown that resveratrol act as a mixed agonist/antagonist on α and β isoforms of estrogen receptors (Bowers et al., 2000). It has thus been suggested that some of the effects of resveratrol could be mediated by the activation of estrogen receptors. However, specific [^{3}H]-resveratrol binding sites characterized in the present study are most unlikely to represent estrogen receptors. In fact, estradiol was unable to compete against specific [^{3}H]-resveratrol binding in rat brain membrane homogenates and was unable to protect primary hippocampal cells against $A\beta_{25-35}$-induced toxicity. Resveratrol was also shown to compete for the aryl hydrocarbon receptor (AhR) with an apparent affinity of 200-1000 nM (Casper et al., 1999); EGCG behaving as an antagonist on this receptor (Palermo et al., 2003). However, it was recently shown that EGCG does not bind directly to AhR, but rather to the chaperone protein, hsp90 (Palermo et al., 2005). Moreover, AhR are cytosolic receptors whereas specific [^{3}H]-resveratrol binding sites are particularly enriched in plasma membrane PII preparations. Finally, benzo[a]pyrene, a polycyclic aromatic hydrocarbon with high affinity for AhR, was unable to compete for specific [^{3}H]-resveratrol binding and had no neuroprotective effect. Taken together, these data demonstrate that specific [^{3}H]-resveratrol binding sites characterized in our study are distinct from the AhR receptors.

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It has recently been shown that catechin and epicatechin can compete with high affinity (20 nM) for [^{3}H]-testosterone/androgen receptors (Gao et al., 2004). However, epicatechin was rather weak to protect hippocampal cell against Aβ-induced toxicity and almost inactive against specific [^{3}H]-resveratrol binding, excluding the androgen receptor as the putative [^{3}H]-resveratrol binding sites characterized in our study. It has also been proposed that EGCG may directly interact with EGF (Liang et al., 1997) and IGF-1 (Shimizu et al., 2005) receptors to regulate receptor tyrosine kinase pathways and the phosphorylation of downstream targets involved in the proliferation of tumoral cells (Shimizu and Weinstein, 2005). In these studies, EGCG and resveratrol at high concentrations (50 μM) induced cell death, while they were both neuroprotective in our model. It thus appears that specific [^{3}H]-resveratrol binding sites identified here represent distinct molecular entities compared to those involved in the facilitation of apoptotic processes associated with EGF and IGF-1 receptors.

Interestingly, EGCG was recently shown to bind with nM affinity to the 67 kDa laminin receptor (LR) (Tachibana et al., 2004). The 67 kDa LR is the mature form of a gene coding for a 37 kDa LR polypeptide which can exist as homodimer or heterodimer (Landowski et al., 1995). The apparent affinity (40 nM) of EGCG for the 67 kDa LR (Tachibana et al., 2004) is similar to that obtained in the present study against specific [^{3}H]-resveratrol binding. Further studies are now in progress to determine if specific [^{3}H]-resveratrol binding characterized here represents the 67 kDa LR. However, the neuroanatomical distribution of specific [^{3}H]-resveratrol binding sites and their

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enrichment in the choroid plexus argue against the 67 kDa LR being the site recognized by [^{3}H]-resveratrol in our study.

Only few resveratrol analogues such as piceatannol, 3,4,4'-trihydroxy-trans-stilbene, 3,5-dihydroxy-trans-stilbene and 3,5-dimethoxy-4'-hydroxy-trans-stilbene shared with catechin gallate esters (ECGC and ECG) the ability to protect hippocampal neuronal cells against $A\beta_{25-35}$-induced toxicity and to compete for specific [^{3}H]-resveratrol binding, demonstrating the existence of clear structure-activity relationships. Indeed, these data suggest that hydroxyl group and gallic acid are required for the neuroprotective activities of resveratrol analogues and catechin gallate esters, respectively. Removing the hydroxyl group in position 4' of resveratrol resulted in an inactive analogue against specific [^{3}H]-resveratrol binding and $A\beta_{25-35}$-induced toxicity. Additionally, the presence of at least one methoxyl group (O-CH3) in one of the pyran rings of the polyphenols tested here (4-hydroxy-4'-methoxy-trans-stilbene, 4,4'-dihydroxy-3-methoxy-trans-stilbene, 3,5-dihydroxy-4'-methoxy-trans-stilben, 3,5-dimethoxy-4'-hydroxy-trans-strilbene and 3,4'-dihydroxy-4-methoxy-trans-stilbene) resulted in markedly reduced affinities for specific [^{3}H]-resveratrol binding and lower potencies to protect against $A\beta_{25-35}$-induced toxicity. We recently reported that gallic acid shared with catechin gallate esters the ability to block cell death induced by various Aβ peptides (Bastianetto et al., 2006), while gallic acid methyl ester was ineffective (data not shown). The structure-activity relationships reported here should be useful toward the development of more potent and selective neuroprotective polyphenols and catechins.

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Ligand receptor autoradiography revealed that specific [^{3}H]-resveratrol binding sites are widely distributed in the rat brain with highest levels of labeling seen in the choroid plexus and subfornical organ followed by the hippocampus and other areas, suggesting that endothelial cells could be involved in the neuroprotective effects of polyphenols. Interestingly, choroid plexus endothelial cells (as transplanted or added in culture medium) have been shown to be neuroprotective in cultured rat hippocampal neurons and rodent models of neurotoxicity, possibly because of their ability to secrete neurotrophic factors (Watanabe et al., 2005). Additionally, dietary supplementation with the Ginkgo biloba extract EGb 761, a complex extract that contains high amounts of polyphenols and prescribed in Europe for the treatment of Alzheimer's disease (Le Bars et al., 2000), enhanced transthyretin mRNA levels in the rat hippocampus (Watanabe et al., 2001). Transthyretin is a thyroid and retinoic acid transporting protein synthesized by the choroid plexus that has been shown to sequester Aβ protein hence preventing its aggregation and toxicity (Tsuzuki et al., 2000). Thus, polyphenols by acting on specific plasma membrane binding sites could increase the expression of transthyretin, this effect possibly also leading to reduced Aβ toxicity.

In summary, we have shown the presence of specific [^{3}H]-resveratrol plasma membrane binding sites in the rat brain. These specific binding sites are of proteinaceous nature, saturable and of rather high affinity. Most importantly, the apparent affinities of a series of analogues of resveratrol and tea catechins for [^{3}H]-resveratrol binding correlated well with their neuroprotective abilities, suggesting their functional relevance. Experiments

are now in progress to identify more precisely the molecular nature of these specific [^{3}H]-resveratrol plasma membrane binding sites in the rat brain.

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LEGENDS FOR FIGURES

Figure 1. Chemical structures of resveratrol, its analogues and polyphenols investigated in this study.

Figure 2. Saturation isotherms of [^{3}H]-resveratrol binding in rat brain membrane homogenates (PII fraction). Insert is a Scatchard transformation of the isotherm saturation binding experiment. Data represent mean ± SEM of a four experiments, each performed in triplicate.

Figure 3. Comparative competition binding profiles of resveratrol, its derivatives and other polyphenols against specific [^{3}H]-resveratrol binding in rat brain membrane homogenates. Data represent the mean ± SEM of three to four determinations, each performed in triplicate and expressed as the percentage of specific binding.

Figure 4. Photomicrographs (A) and quantitative (B) autoradiographic distribution of specific [^{3}H]-resveratrol binding sites in the rat brain. Nonspecific binding (NS) was determined in the presence of 100 µM resveratrol. Abbreviations; Amy, Amygdala; Cg, cingulate cortex; Ch, Choroid plexus; CPu, caudate putamen; Fr, frontal cortex; Hi, hippocampus; Hyp, hypothalamus; IP, interpeduncular nuclei; LS, lateral septum; Par, parietal cortex; Pir, piriform cortex; SFO, subfornical organ; Th, thalamus.

Figure 5. Comparative affinities and potencies of various resveratrol analogues and polyphenols to compete for specific [^{3}H]-resveratrol binding and protect against $A\beta_{25-35}$-induced toxicity in primary rat hippocampal cell cultures.

Table 1. Specific [^{3}H]-resveratrol binding in various subcellular fractions from rat brain homogenates.

Fraction	Specific [^{3}H]-resveratrol bound (DPM)
PI. Nucleus and cell debris	9573
PII. Plasma membrane	15572
PII pellet after pre-treatment with	
Benzonase (50 U/ml at room temperature)	13770
Boiling (100^{o}C for 10 min)	2853
Trypsin (0.25% at 37^{o}C for 10 min)	3178
SII. Soluble proteins	915

Binding was performed using a concentration of 20 nM [^{3}H]-resveratrol. Non-specific binding was residual binding seen in presence of 100 μM resveratrol. DPM represent desintegration per minute.

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Table 2. Comparison between apparent affinity for specific [^{3}H]-resveratrol binding and neuroprotective effect against $A\beta_{25-35}$-induced toxicity of resveratrol derivatives and other polyphenols.

Compound	Apparent affinity (Ki nM)	Neuroprotective activity (EC_{50} μM)
Resveratrol	102 ± 22	13 ± 3
Trans-stilbene	> 10000	>> 50
Piceatannol	830 ± 75	11 ± 2
Trans-4-stilbenemethanol	> 10000	>> 50
Diethylstilbestrol	> 10000	>> 50
3,4-dihydroy-trans-stilbene	> 10000	>> 50
3,5-dihydroy-trans-stilbene	300 ± 45	17 ± 3
3,4,5-trihydroxy-trans-stilbene	> 10000	>> 50
3,4,4'-trihydroxy-trans-stilbene	1370 ± 240	6 ± 1
4,4'-dihydroxy-trans-stilbene	> 10000	>> 50
4-hydroxy-4'-methoxy-trans-stilbene	> 10000	> >50
4,4'-dihydroxy-3-methoxy-trans-stilbene	> 10000	>> 50
4-hydroxy-trans-stilbene	> 10000	>> 50
3,5-dihydroxy-4'-methoxy-trans-stilbene	> 10000	>> 50
3,5-dimethoxy-4'-hydroxy-trans-stilbene	2500 ± 360	26 ± 4
3,4'-dihydroxy-4-methoxy-trans-stilbene	> 10000	>> 50
(-)-Epicatechin	> 10000	81
(-)-Epicatechin gallate	25 ± 9	7 ± 1
(-)-Epigallocatechin	> 10000	63
(-)-Epigallocatechin gallate	45 ± 18	5 ± 1
Estradiol	> 10000	>> 50
Benzo[a]pyrene	> 10000	>> 50

Data are mean ± SEM of three to four determinations. Ki represents the concentration of competitor needed to inhibit 50% specifically bound [^{3}H]-resveratrol. EC_{50} represents the concentration of compound required to block 50% of cell death induced by $Aß_{25-35}$ (25 μM).

Figure 1

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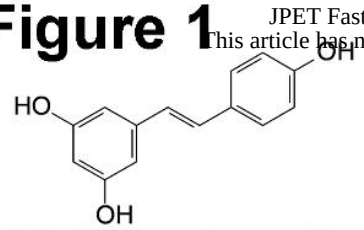

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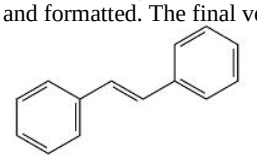

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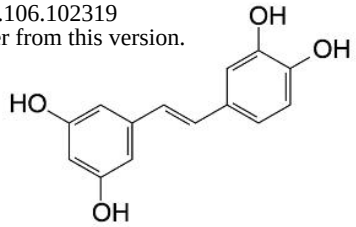

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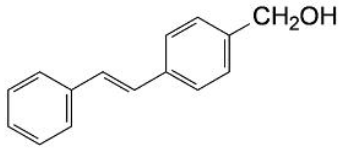

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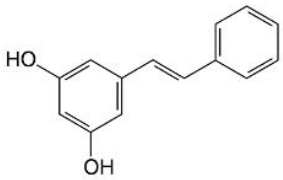

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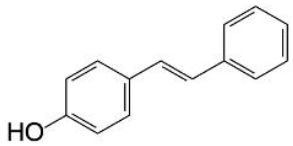

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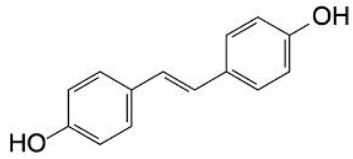

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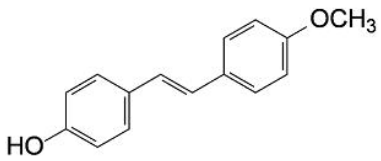

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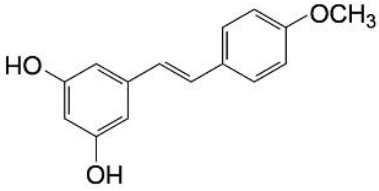

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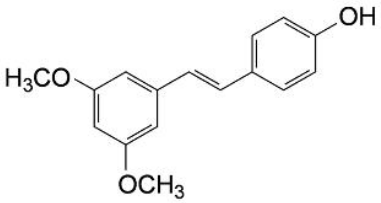

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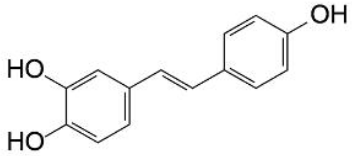

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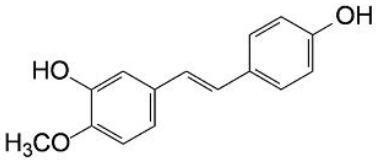

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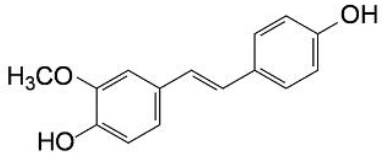

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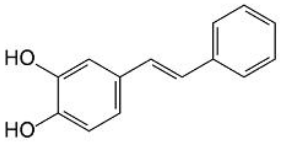

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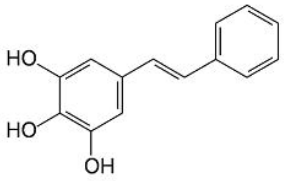

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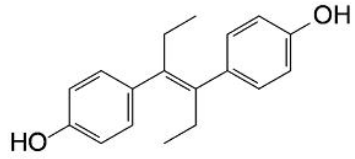

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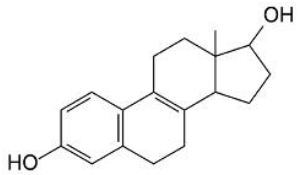

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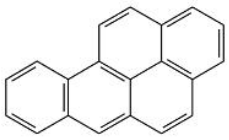
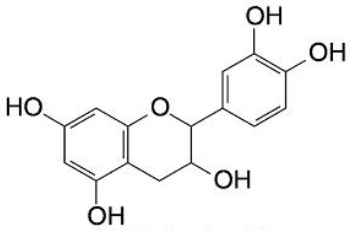


benzo[a]pyrene



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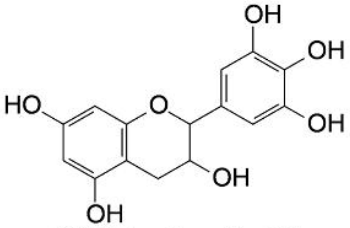

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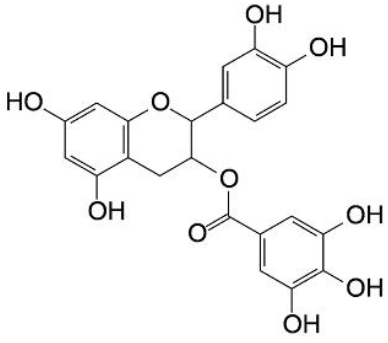

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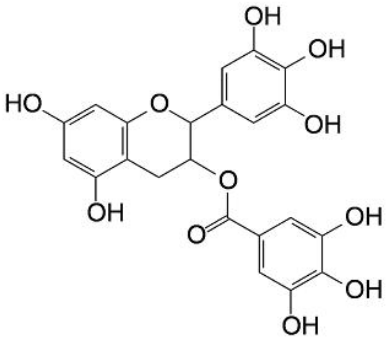

(-)-Epigallocatechin gallate

Figure 2

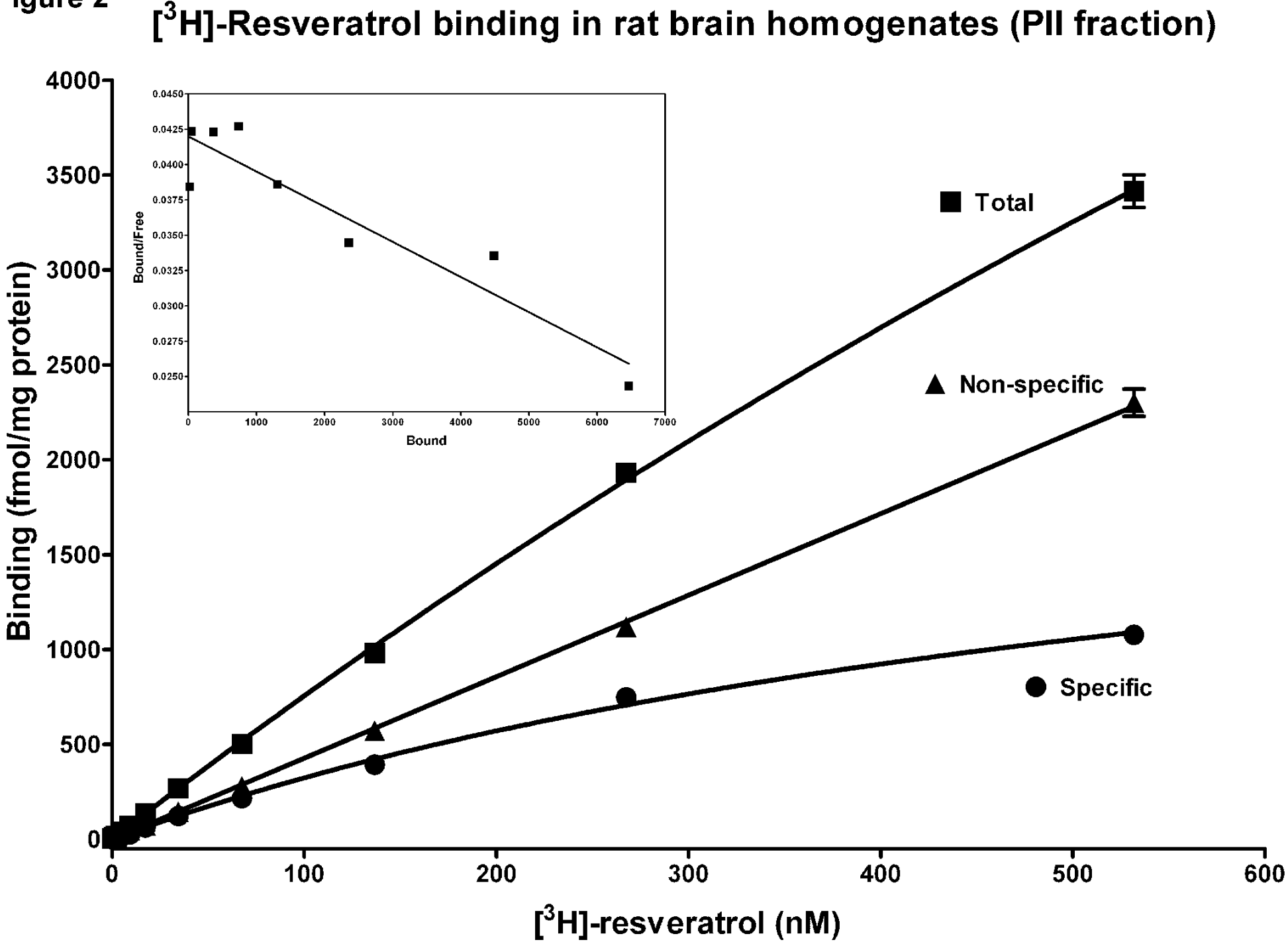

Figure 3

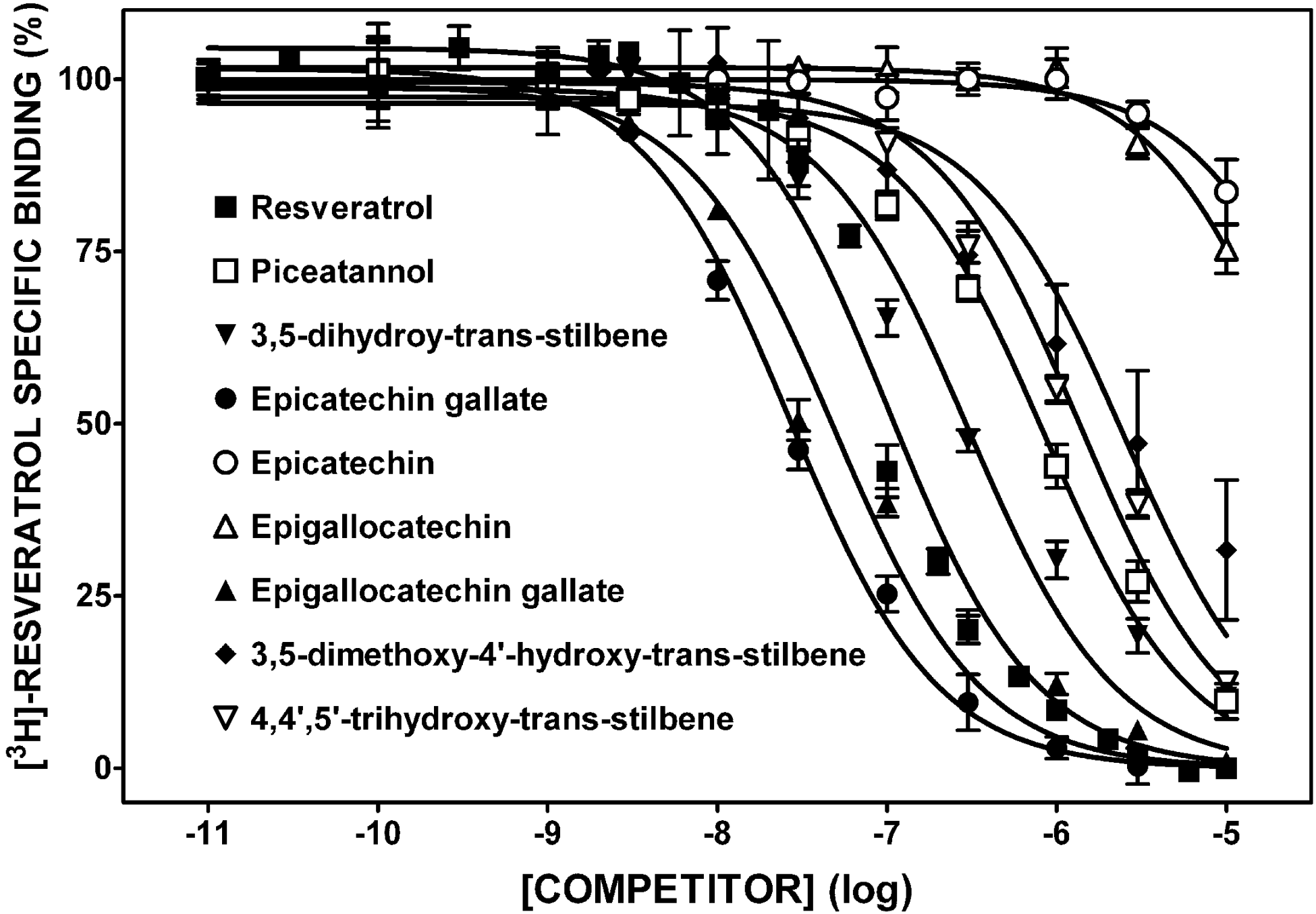

Figure 4