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Biochemical and Biophysical Research Communications 344 (2006) 688–695
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Inhibitory effects of *cis*- and *trans*-resveratrol on noradrenaline and 5-hydroxytryptamine uptake and on monoamine oxidase activity ☆

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Received 27 February 2006
Available online 17 April 2006

Abstract

This study investigated for the first time the potential effects of *cis*- and *trans*-resveratrol (*c*-RESV and *t*-RESV) on noradrenaline (NA) and 5-hydroxytryptamine (5-HT) uptake by synaptosomes from rat brain, on 5-HT uptake by human platelets, and on monoamine oxidase (MAO) isoform activity. Both *c*-RESV and *t*-RESV (5–200 μM) concentration-dependently inhibited the uptake of [^{3}H]NA and [^{3}H]5-HT by synaptosomes from rat brain and the uptake of [^{3}H]5-HT by human platelets. In both experimental models, *t*-RESV was slightly more efficient than *c*-RESV. Furthermore, in synaptosomes from rat brain, the RESV isomers were less selective against [^{3}H]5-HT uptake than the reference drug fluoxetine (0.1–30 μM). On the other hand, both *c*-RESV and *t*-RESV (5–200 μM) concentration-dependently inhibited the enzymatic activity of commercial (human recombinant) MAO isoform (MAO-A and MAO-B) activity, *c*-RESV being slightly less effective than *t*-RESV. In addition, both RESV isomers were slight but significantly more selective against MAO-A than against MAO-B. Since the principal groups of drugs used in the treatment of depressive disorders are NA/5-HT uptake or MAO inhibitors, under the assumption that the RESV isomers exhibit a similar behaviour in humans in vivo, our results suggest that these natural polyphenols may be of value as structural templates for the design and development of new antidepressant drugs with two important biochemical activities combined in the same chemical structure: NA/5-HT uptake and MAO inhibitory activity.

Keywords: *cis*-Resveratrol; *trans*-Resveratrol; Noradrenaline and 5-hydroxytryptamine uptake; Human recombinant MAO isoforms; Human platelets; Rat brain synaptosomes

Resveratrol (3,4′,5-trihydroxystilbene, RESV, Fig. 1) is a natural phenolic compound that is present in wines and that exists as *cis*- and *trans*-isomers [the *c*-RESV or (*Z*)-RESV diasteromer and *t*-RESV or (*E*)-RESV diasteromer, respectively], facilitated by the double bond in its chemical structure [1–4].

To date, most pharmacological studies have considered the *trans* isomer, which has been suggested to be one of the principal wine components responsible for the cardioprotective effects attributed to the long-term moderate consumption of wine [1,5–9].

In in vitro, ex vivo, and in vivo experiments *t*-RESV has shown a number of biological activities including anti-inflammatory and anticarcinogenic properties [1,2,9–13].

In contrast, much less is known about the pharmacological activity of the *cis*-isomer (*c*-RESV), possibly as a result of the fact that *c*-RESV (unlike the *trans* isomer) is not available commercially. The few comparative studies reported in the scientific literature on the biological effects of *cis*- versus *t*-RESV have generally demonstrated only quantitative, not qualitative differences, in the activities of the two forms (for more details, see [3,14]).

In a preliminary and comparative study of the potential effects of the RESV isomers on platelet function (unpublished results; for a review, see [14]), we have recently observed that these natural polyphenols, like a number of

☆ *Abbreviations:* ANOVA, analysis of variance; *c*-RESV, resveratrol, *cis* isomer; DMSO, dimethyl sulfoxide; 5-HT, 5-hydroxytryptamine; MAO, monoamine oxidase; NA, noradrenaline; PRP, platelet-rich plasma; RESV, resveratrol; *t*-RESV, resveratrol, *trans* isomer.

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doi:10.1016/j.bbrc.2006.03.190

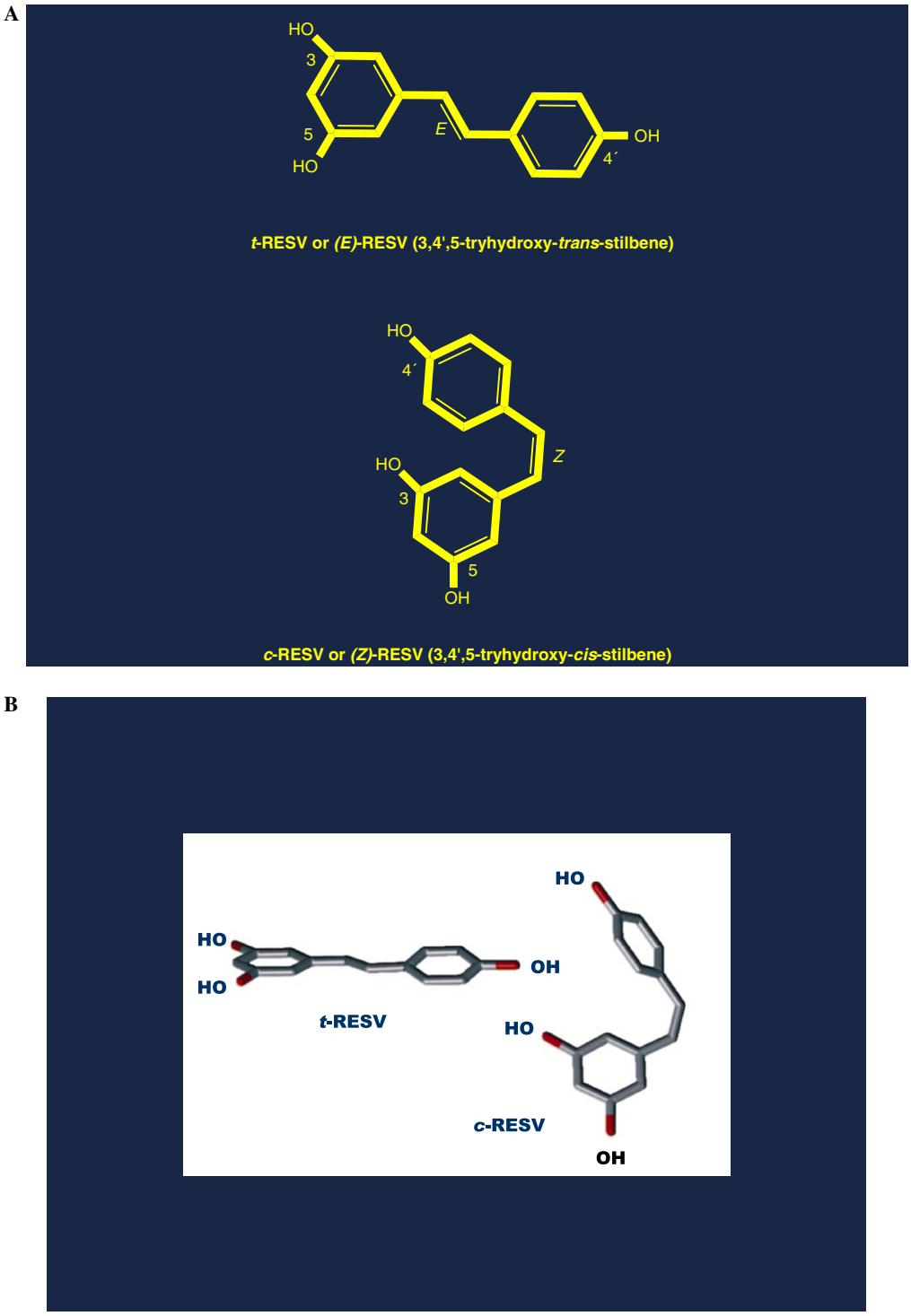

Fig. 1. Chemical structures (A) and 3D molecular configurations (B) of the *cis*- and *trans*-isomers of RESV.

antidepressant drugs [15–17], inhibit the uptake of 5-hydroxytryptamine (5-HT) by human platelets.

On the other hand, it is well known that another class of drugs used to treat major depressive disorders are the inhibitors of monoamine oxidase (MAO) activity [15,17]. To date, however, the effects of RESV isomers on MAO isoform (MAO-A and MAO-B) activity have not been studied. Only Zhou et al. [18], in a preliminary structure–activity relationship study of a number of stilbenoids, have previously shown that *t*-RESV exhibits a selective inhibitory effect on MAO-A activity but not on MAO-B.

Bearing in mind the above considerations, we here report the first study of the potential effects of the RESV isomers on the uptake of [^{3}H]noradrenaline ([^{3}H]NA) and [^{3}H]5-HT, using two traditional models widely used in the evaluation of the effects of new antidepressant drugs

[19,20]: a central model (synaptosomes from rat brain) and a peripheral model (human platelets). In addition, we also report the potential effects of both *c*-RESV and *t*-RESV on human recombinant MAO isoform activity.

Materials and methods

Animals. The animals used throughout this study [male Sprague–Dawley rats (Iffa-Credo), purchased from Criffa (Barcelona, Spain)] were housed, cared for, and acclimatized (before the experiments) as previously indicated [21].

Ethical approval. All experiments were carried out in accordance with European regulations on the protection of animals (Directive 86/609), the Spanish *Real Decreto* 1201/2005 and/or the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996). In addition, all experimental protocols were approved by the Institutional Animal Care and Use Committee of the University of Santiago de Compostela (Spain), and conducted humanely.

Preparation of synaptosomes from rat brain. Crude synaptosomal fractions from rat brain were prepared following the standard procedure described by Hajos [22] with some modifications. Briefly, male Sprague–Dawley rats were sacrificed by exposure to CO_2 and subsequent decapitation. Whole brains were rapidly removed, placed in a Petri dish with ice-cold sucrose solution (0.32 M, pH 7.4), and cleared of cerebral cortex and cerebellum. Remaining brain tissues (approximately 1 g) were then diluted 10-fold with ice-cold sucrose solution (0.32 M, pH 7.4) and homogenized using a glass Potter–Elvehjem tube with a Teflon pestle (10 up and down strokes). The homogenate solution was centrifuged at 1500*g* for 10 min at 4 °C in a refrigerated centrifuge 4K10 (Sigma, Osterade, Germany). The pellet was discarded and the supernatant was subsequently centrifuged at 20,000*g* for 20 min at 4 °C in a centrifuge J2-MI (Beckman Instruments, Palo Alto, California, USA). The resultant pellet of this centrifugation was gently homogenized in a glass Potter-type homogenizer with a manual glass pestle, resuspended in 20 ml of ice-cold Krebs–Henseleit solution [previously oxygenated with carbogen (95% O_2 + 5% CO_2) at room temperature (20 ± 2 °C)] of the following composition (mM): NaCl 119, KCl 4.7, $MgSO_4{\cdot}7H_2O$ 1.2, KH_2PO_4 1.2, $CaCl_2{\cdot}2H_2O$ 1.9, $NaHCO_3$ 25, and glucose 11 (pH 7.4), and then used as a crude synaptosome preparation in the corresponding experiments. The size of the synaptosomes was measured in a Zetasizer (NanoZS, Malver Instruments, Wencestershire, UK) and was 2,651 ± 74 nm.

The protein concentration in the crude synaptosome preparation (approximately of 1 mg/ml) was measured by the method of Bradford [23], using a protein assay kit from Bio-Rad Laboratories (Alcobendas, Spain).

Determination of monoamine ([^{3}H]NA/[^{3}H]5-HT) uptake by synaptosomes from rat brain. Rat brain synaptosomes were prepared according to the method described in the above section. The [^{3}H]NA/[^{3}H]5-HT uptake was evaluated according to the general method described by Bolden-Watson and Richelson [24] with some modifications.

Test solutions of 0.5 ml containing the RESV isomers (*c*-RESV or *t*-RESV) in various concentrations (5–200 μM) and the synaptosome suspension (final concentration: 0.6 mg protein/ml) in Krebs–Henseleit solution were incubated for 15 min at 37 °C in appropriate polypropylene tubes (Deltalab, Barcelona, Spain) placed in a shaking water bath Unitronic 320 OR (Selecta, Barcelona, Spain). After this incubation period, monoamine (NA/5-HT) uptake was initiated by the addition of 6.36 nM [^{3}H]5-HT (specific activity: 15.7 Ci/mmol) and 43.64 nM non-radioactive 5-HT (total 5-HT: 50 nM, final concentration; 100,000 dpm/tube) or 7.36 nM [^{3}H]NA (specific activity: 12 Ci/mmol) and 42.64 nM non-radioactive NA (total NA: 50 nM, final concentration; 100,000 dpm/tube), and continued at 37 °C for 15 min. NA/5-HT uptake was stopped by immediate placement of assay tubes into an ice-bath, followed by the addition in each sample of 3 ml ice-cold Krebs–Henseleit solution. Test tube contents were rapidly poured over Whatman GF/B filters (Whatman, Clifton, NJ, USA) presoaked in polyethyleneimine [0.1% (v/v)] to reduce non-specific binding and were filtered in a cell harvester M-30 (Biomedical Research and Development Laboratories, Gaithersburg, USA). Filters were washed five times (3 ml each) in ice-cold Krebs–Henseleit solution, dried for 1 h at 60 °C in a Memmert 200 oven (Afora, Barcelona, Spain), and placed in 20 ml scintillation vials. After, 6 ml of liquid scintillation cocktail (Ecoscint A, National Diagnostics, Hessle Hull, UK) was added to each vial and the radioactivity of the samples (retained in the filters) was measured in a liquid scintillation counter Beckman LS6500 (Beckman Instruments, Fullerton, California, USA), following appropriate standard control procedures.

Control experiments were performed in parallel by replacing the RESV isomers with appropriate dilutions of the vehicle [dimethyl sulfoxide (DMSO)].

Non-specific uptake (that is, non-specific transport and non-specific binding of [^{3}H]5-HT or [^{3}H]NA) was determined by running parallel assays at 0 °C, a temperature at which monoamine transporters are thought to be inactive. Specific [^{3}H]5-HT or [^{3}H]NA uptake was indirectly estimated by subtracting the non-specific uptake from the total uptake determined at 37 °C.

In some experiments, the sensitivity of the method was evaluated by measuring the influence of several well-known reference inhibitors of NA/5-HT uptake under the conditions described above.

Preparation of washed platelets. Human platelets were isolated by centrifugation from buffy coats obtained from the Centro de Transfusión de Galicia (Santiago de Compostela, Spain) and prepared as we have previously described [25]. Briefly, buffy coat was diluted 1:1 with washing buffer of the following composition (mM): NaCl 120, KCl 5, trisodium citrate 12, glucose 10, and sucrose 12.5 (pH 6), and then centrifuged at 390*g* for 9 min in a centrifuge Omnifuge 2.0 RS (Heraeus Sepatech, Osterade, Germany) at 25 °C to obtain platelet rich plasma (PRP). PRP was centrifuged at 1000*g* for 18 min at 25 °C in a centrifuge Omnifuge 2.0 RS (Heraeus Sepatech, Osterade, Germany) and the resulting platelet pellet was recovered, diluted to 8 ml with washing buffer, and centrifuged again at 1000*g* for 15 min at 25 °C. Finally, the platelet pellet recovered from this step was resuspended in a modified Tyrode–Hepes buffer [composition (mM): Hepes 10, NaCl 140, KCl 3, $MgCl_2$ 0.5, $NaHCO_3$ 5, and glucose 10, pH 7.4], providing a concentration of 2.5–3.5 × 10^8 platelets/ml. The calcium concentration in the extracellular medium was 2 mM. The protein content of this platelet suspension was of approximately 0.5 mg/ml, determined using the method of Bradford [23].

Determination of monoamine [^{3}H]NA/[^{3}H]5-HT uptake by human platelets. Washed platelets were prepared as described above. 0.5 ml of the platelet suspension containing the RESV isomers (*c*-RESV or *t*-RESV) in various concentrations (ranging from 5 to 200 μM) was incubated for 15 min at room temperature in appropriate Eppendorf vials. After this incubation period, 5-HT uptake was initiated by the addition of 19.66 nM [^{3}H]5-HT (specific activity: 15.7 Ci/mmol) and 5.34 nM non-radioactive 5-HT (total 5-HT: 25 nM, final concentration; 100,000 dpm/tube), and continued for 15 min.

An aliquot of 100 μl was used to count directly the total radioactivity (see below). The remaining 400 μl were added to Eppendorf vials containing 200 μl of formaldehyde at 0 °C (1% v/v, final concentration) to stop the [^{3}H]5-HT uptake. This sample was then centrifuged for 10 min at 2000*g* in a centrifuge (Microfuge™, Beckman Instruments, Palo Alto, California, USA). An aliquot of the resulting supernatant was placed in 5 ml scintillation vials and the radioactivity was measured by liquid scintillation in a Beckman LS6500 counter (Beckman Instruments, Fullerton, California, USA) after the addition of 3 ml of scintillation cocktail (Ecoscint A, National Diagnostics, Hessle Hull, UK) in each tube.

The amount of [^{3}H]5-HT incorporated into platelets was calculated by the difference between the total radioactivity present in the sample and the radioactivity present in the supernatant.

Similarly to studies on [^{3}H]NA/[^{3}H]5-HT uptake by synaptosomes from rat brain, control experiments were performed by replacing the RESV isomers with appropriate dilutions of the vehicle (DMSO).

In some tests, the validity of the method was checked by measuring the influence of various reference inhibitors of monoamine uptake under the experimental conditions given in the above paragraphs.

Determination of MAO isoform activity. The potential effects of the RESV isomers on MAO activity were investigated by measuring their effects on the production of hydrogen peroxide H_2O_2 (and, therefore, of resorufin) from *p*-tyramine, using the Amplex® Red MAO assay kit (Molecular Probes, Eugene, Oregon, USA) and microsomal MAO isoforms prepared from insect cells (BTI-TN-5B1-4) infected with recombinant baculovirus containing cDNA inserts for human MAO-A or MAO-B (Sigma–Aldrich Química S.A., Alcobendas, Spain) (for more details, see Discussion).

In our experiments, MAO activity was evaluated by the above-mentioned method, following the general procedure previously described [26] with several modifications and using a Aminco-Bowman® Series 2 Spectrometer (ThermoSpectronic, Rochester, NY, USA) for measuring the generation of resorufin.

Briefly, 0.3 ml of sodium phosphate buffer (0.05 M, pH 7.4) containing the test drugs (RESV isomers or reference inhibitors) in various concentrations and pure MAO-A or MAO-B required to oxidize (in the control group) 165 pmol of *p*-tyramine/min (MAO-A: 1.1 μg; specific activity: 22 nmol of *p*-tyramine oxidized to *p*-hydroxyphenylacetaldehyde per min per mg protein; MAO-B: 7.5 μg; specific activity: 150 nmol of *p*-tyramine transformed per min per mg protein) were incubated for 15 min at 37 °C in appropriate semi-micro PMMA cuvettes of 1.5 ml (Plastibrand, Wertheim, Germany) already placed into the dark fluorimeter chamber. After this incubation period, reaction was started by adding (final concentrations) 200 μM Amplex Red reagent, 1 U/ml horseradish peroxidase, and 1 mM *p*-tyramine as a common substrate for both MAO-A and MAO-B.

The production of H_2O_2 and, consequently, of resorufin was quantified at 37 °C in an Aminco-Bowman® Series 2 Spectrometer (ThermoSpectronic, Rochester, NY, USA) on the basis of fluorescence generated (excitation 545 nm, emission 590 nm) over a 15 min period, a period in which fluorescence increased linearly from the beginning.

Control experiments were carried out simultaneously by replacing the test drugs (RESV isomers or reference inhibitors) with appropriate dilutions of the vehicles. In addition, the possible capacity of the above-mentioned test drugs to directly react with Amplex Red reagent was determined by adding these drugs to solutions containing only the Amplex Red reagent in a sodium phosphate buffer.

The specific fluorescence emission (used to obtain the final results) was calculated after subtraction of background activity which was determined from vials containing all components with the exception of the MAO isoforms, which were replaced by a sodium phosphate buffer solution.

Data presentation and statistical analysis. Unless otherwise specified, results shown in the text and tables are expressed as means ± SEM. Significant differences between two means ($p < 0.05$ or $p < 0.01$) were determined by one-way analysis of variance (ANOVA) followed by Dunnett's post hoc test.

[^{3}H]NA and [^{3}H]5-HT uptake by human platelets and by synaptosomes from rat brain were determined as: [^{3}H]NA or [^{3}H]5-HT uptake [pmol/mg of protein] = (dpm in platelets or synaptosomes/mg of protein) × (pmol [^{3}H]NA and [^{3}H]5-HT in 1 L of solution/dpm in 1 L of solution). Note that the numerator of the second factor in this expression is the concentration of [^{3}H]NA or [^{3}H]5-HT, not the total NA or 5-HT concentration. In these experiments, the inhibitory activity of the tested compounds (RESV isomers and reference inhibitors) is expressed as IC_{50}, i.e., the concentration of these compounds required to reduce by 50% the [^{3}H]NA or [^{3}H]5-HT uptake, estimated by least-squares linear regression, using the program Origin™ 5.0 (Microcal Software, Northampton, USA), with X = log molar concentration of tested compound and Y = pharmacological response (% decrease in the control [^{3}H]NA or [^{3}H]5-HT uptake) obtained with each concentration. This regression was performed using data obtained with 4–6 different concentrations of tested compound which inhibited the [^{3}H]NA or [^{3}H]5-HT by between 20% and 80%. In addition, the corresponding [^{3}H]5-HT uptake selectivity ratios [IC_{50} ([^{3}H]NA)]/[IC_{50} ([^{3}H]5-HT)] were calculated.

In the experiments designed to study the possible effects of RESV isomers on MAO isoform enzymatic activity, the variation of fluorescence per unit of time (fluorescence arbitrary U) and, indirectly, the rate of H_2O_2 production and, therefore, the pmol/min of resorufin produced in the reaction between H_2O_2 and Amplex Red reagent was evaluated. For this purpose, several concentrations of resorufin were used to prepare a standard curve with X = pmol resorufin and Y = fluorescence arbitrary U. Note that the value of resorufin production is similar to the pmol of *p*-tyramine oxidized to *p*-hydroxyphenylacetaldehyde per min since the stoichiometry of the reaction (*p*-tyramine oxidized by MAO isoforms/resorufin produced) is 1:1.

In these experiments, IC_{50} values for the tested drugs (RESV isomers and reference inhibitors) were assessed (as described above) by least-squares linear regression with X = log of tested compound molar concentration and Y = the corresponding percentage of inhibition of control resorufin production obtained with each concentration. In addition, the MAO-A selectivity ratios [IC_{50} (MAO-B)]/[IC_{50} (MAO-A)] were calculated.

Drugs, chemicals, and radioisotopes. The drugs used in the experiments were *t*-RESV, (±)-fluoxetine hydrochloride, imipramine hydrochloride, (−)-NA bitartrate, 5-HT hydrochloride, (±)-nisoxetine hydrochloride, citalopram hydrobromide, *R*-(−)-deprenyl hydrochloride, and iproniazid phosphate (all purchased from Sigma–Aldrich, Spain), resorufin sodium salt, clorgyline hydrochloride, *p*-tyramine hydrochloride, and horseradish peroxidase (supplied in the Amplex® Red MAO assay kit from Molecular Probes), and *c*-RESV. This isomer of RESV was prepared at the Departamento de Química Orgánica (Universidad de Santiago de Compostela) following the method previously described [27].

The radioisotopes *L*-[7,8-^{3}H]noradrenaline ([^{3}H]NA, 1 μCi/μl, specific activity: 12 Ci/mmol) and 5-hydroxy[G-^{3}H]tryptamine creatinine sulphate ([^{3}H]5-HT, 1 μCi/μl, specific activity: 15.7 Ci/mmol) were from Amersham Biosciences Europe, Madrid, Spain.

Appropriate dilutions of the above drugs were prepared every day immediately before use in deionized water from the following concentrated stock solutions kept at −20 °C: *t*-RESV and *c*-RESV (0.1 M) in DMSO; (−)-NA, 5-HT, (±)-fluoxetine, citalopram, imipramine, (±)-nisoxetine, *R*-(−)-deprenyl, iproniazid, resorufin, clorgyline, *p*-tyramine, and horseradish peroxidase (0.1 M) in deionized water. Sodium bisulfite (0.2% w/v) was added to the NA stock solution to prevent oxidation.

Due to the photosensitivity of the RESV isomers and some chemicals (e.g., Amplex Red reagent), all experiments in which these compounds were used were performed in the dark. In all assays, neither deionized water (Milli-Q®, Millipore Ibérica S.A., Madrid, Spain) nor appropriate dilutions of the vehicle used (DMSO) had significant pharmacological effects.

The specific chemicals and materials used in the different tests were purchased from suppliers indicated in the corresponding sections.

All the other chemicals, including the reagents used in the preparation of the different buffers and physiological solutions, were of the best quality commercially available.

Results

Effects of RESV isomers on the uptake of [^{3}H]NA and [^{3}H]5-HT by rat brain

Both [^{3}H]NA and [^{3}H]5-HT were accumulated into synaptosomes from rat brain. In control crude synaptosomal fractions, the [^{3}H]NA uptake was 12.3 ± 0.6 pmol [^{3}H]NA/mg protein ($n = 20$) whereas the [^{3}H]5-HT uptake was 10.6 ± 0.4 pmol [^{3}H]5-HT/mg protein ($n = 20$).

Imipramine (0.3–100 μM), a reference non-selective inhibitor of NA and 5-HT uptake, concentration-dependently and non-selectively inhibited synaptosomal accumulation of [^{3}H]NA and [^{3}H]5-HT since the corresponding IC_{50} value for inhibiting [^{3}H]NA uptake did not present significant differences ($p > 0.05$) with respect to the IC_{50} for decreasing [^{3}H]5-HT uptake (Fig. 2 and Table 1).

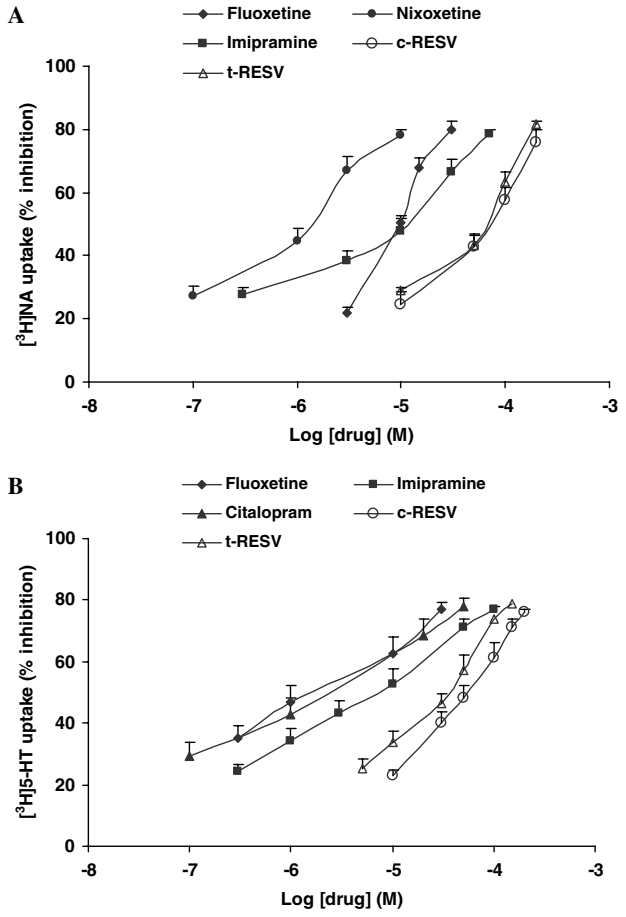

Fig. 2. Concentration–response curves for the inhibitory effects of test drugs (RESV isomers and reference inhibitors) on the uptake of [^{3}H]NA (A) and [^{3}H]5-HT (B) by synaptosomes from rat brain. Each point represents the mean value ± SEM (indicated by vertical bars) from five experiments.

Table 1
IC_{50} values (μM) and [^{3}H]5-HT uptake selectivity ratios [IC_{50} ([^{3}H]NA)]/[IC_{50} ([^{3}H]5-HT)] for the inhibitory effects of test drugs (RESV isomers and reference inhibitors) on the uptake of [^{3}H]NA and [^{3}H]5-HT by synaptosomes from rat brain

DRUG	[^{3}H]NA uptake (IC_{50})	[^{3}H]5-HT uptake (IC_{50})	Ratio
Fluoxetine	10.12 ± 1.02*	2.52 ± 0.59	4.02
Nisoxetine	1.10 ± 0.29*	>100[a]	<0.01
Imipramine	11.77 ± 1.46	8.54 ± 0.69	1.38
Citalopram	>100*,[a]	15.81 ± 4.21	>6.33
c-RESV	79.32 ± 2.68#	51.55 ± 1.62	1.54
t-RESV	69.70 ± 2.51#	32.49 ± 1.25	2.15

Each value is the mean ± SEM from five experiments (in each separate experiment, the corresponding sample was assayed in triplicate). Level of statistical significance: $^{\#}p < 0.05$ or $^{*}p < 0.01$ versus the corresponding IC_{50} values of [^{3}H]5-HT uptake, as determined by ANOVA/Dunnett's.
[a] IC_{50} value > highest concentration tested, 100 μM.

Furthermore, the [^{3}H]5-HT uptake selectivity ratio [IC_{50} ([^{3}H]NA)]/[IC_{50} ([^{3}H]5-HT)] was 1.38, a value very close to 1.

In contrast, citalopram (0.1–100 μM), a well-known reference selective inhibitor of 5-HT uptake, selectively inhibited the [^{3}H]5-HT uptake by synaptosomes from rat brain and had no effect on [^{3}H]NA uptake at concentrations from 0.1 to 100 μM (the highest concentration tested) (Fig. 2 and Table 1).

In this experimental model, however, fluoxetine (0.2–30 μM), another well-known selective inhibitor of 5-HT uptake, as well as the RESV isomers (5–200 μM) only exhibited a slight selectivity against [^{3}H]5-HT uptake since the corresponding IC_{50} values for decreasing this uptake were slight but significantly lower than the corresponding IC_{50} values for reducing [^{3}H]NA uptake (Fig. 2 and Table 1). In addition, *t*-RESV was slight, but significantly more effective than *c*-RESV since the corresponding IC_{50} values for decreasing [^{3}H]5-HT uptake and [^{3}H]NA uptake were significantly lower ($p < 0.05$).

On the other hand, nisoxetine (0.1–30 μM), a selective NA uptake inhibitor, selectively inhibited the [^{3}H]NA uptake and had no effect on [^{3}H]5-HT uptake at concentrations from 0.1 to 30 μM and at the highest concentration tested (100 μM) (Fig. 2 and Table 1).

Effects of RESV isomers on the uptake of [^{3}H]5-HT by human platelets

Unlike synaptosomes from rat brain, human platelets only accumulated [^{3}H]5-HT but not [^{3}H]NA. Basal uptake of [^{3}H]5-HT by the control human platelets was 58.9 ± 2.1 pmol [^{3}H]5-HT/mg protein ($n = 20$) after an incubation period with [^{3}H]5-HT of 15 min.

Imipramine (3–20 nM), citalopram (2–65 nM), and fluoxetine (3–30 nM) concentration-dependently inhibited this uptake (Fig. 3 and Table 2). For all these reference inhibitors tested, the corresponding IC_{50} values for decreasing [^{3}H]5-HT uptake were significantly lower ($p < 0.01$) than the corresponding IC_{50} values for inhibiting [^{3}H]5-HT uptake by synaptosomes from rat brain (Tables 1 and 2).

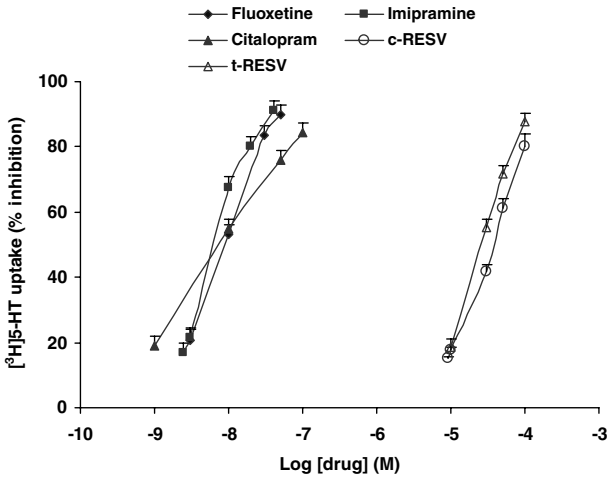

Fig. 3. Concentration–response curves for the inhibitory effects of test drugs (RESV isomers and reference inhibitors) on the uptake of [^{3}H]5-HT by human platelets. Each point represents the mean value ± SEM (indicated by vertical bars) from five experiments.

Table 2
IC_{50} values (μM) for the inhibitory effects of test drugs (RESV isomers and reference inhibitors) on the uptake of [^{3}H]5-HT by human platelets

Drug	[^{3}H]5-HT uptake (IC_{50})
Fluoxetine	$7.81 \times 10^{-3} \pm 0.36$
Imipramine	$8.22 \times 10^{-3} \pm 0.76$
Citalopram	$7.57 \times 10^{-3} \pm 0.44$
c-RESV	$37.41 \pm 1.47^{\#}$
t-RESV	24.30 ± 1.83

Results are means ± SEM from five experiments (in each separate experiment, the corresponding sample was assayed in triplicate). Level of statistical significance: $^{\#}p < 0.05$ versus the corresponding IC_{50} value of *t*-RESV.

On the other hand, both *c*-RESV and *t*-RESV (5–200 μM) concentration-dependently inhibited the uptake of [^{3}H]5-HT by human platelets, *t*-RESV being slight, but significantly more effective than *c*-RESV (Fig. 3 and Table 2). In addition, the corresponding IC_{50} values for decreasing this uptake did not present significant differences ($p > 0.05$) with respect to the IC_{50} for reducing [^{3}H]5-HT uptake by synaptosomes from rat brain (Tables 1 and 2).

Effects of RESV isomers on human isoform MAO activity

The test drugs (RESV isomers and reference inhibitors) themselves were unable to directly react with Amplex Red reagent. On the other hand, the control activity of MAO-A and MAO-B (using *p*-tyramine as common substrate for both isoforms) was 165 ± 2 pmol of *p*-tyramine oxidized to *p*-hydroxyphenylacetaldehyde/min ($n = 20$).

Iproniazid (1–50 μM), a reference non-selective inhibitor of MAO-A and MAO-B, concentration-dependently and non-selectively inhibited this control enzymatic activity (Fig. 4 and Table 3). The corresponding IC_{50} values and the MAO-A selectivity ratio [IC_{50} (MAO-B)]/[IC_{50} (MAO-A)] are shown in Table 3.

In contrast, clorgyline (0.5–20 nM) was a selective inhibitor of MAO-A (MAO-A selectivity ratio: 8165.06) whereas deprenyl (10–100 nM) was a selective inhibitor of MAO-B (MAO-A selectivity ratio: 0.000414) (Table 3).

On the other hand, both *c*-RESV and *t*-RESV (5–200 μM) concentration-dependently inhibited the enzymatic activity of commercial (human recombinant) MAO-A and MAO-B (Fig. 4 and Table 3). *c*-RESV was slight, but significantly less effective than *t*-RESV since the corresponding IC_{50} values for decreasing MAO-A and MAO-B activity were significantly higher ($p < 0.05$). In addition, both RESV isomers were slight but significantly more selective inhibitors against MAO-A than against MAO-B (Table 3).

Discussion

This is the first comparative study of the potential effects of the RESV isomers on the uptake of [^{3}H]NA and [^{3}H]5-HT by synaptosomes from rat brain, on [^{3}H]5-HT uptake by human platelets, and on human recombinant MAO isoform activity.

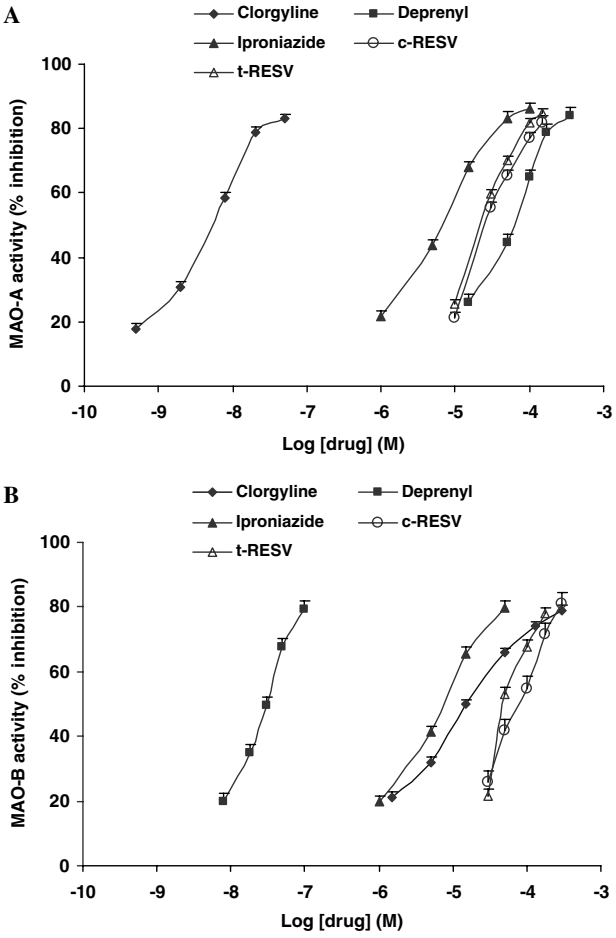

Fig. 4. Concentration–response curves for the inhibitory effects of test drugs (RESV isomers and reference inhibitors) on the enzymatic activity of human recombinant MAO-A (A) or MAO-B (B) (for more details, see text). Each point represents the mean value ± SEM (indicated by vertical bars) from five experiments.

Table 3
IC_{50} values (μM) and MAO-A selectivity ratios [IC_{50} (MAO-B)]/[IC_{50} (MAO-A)] for the inhibitory effects of test drugs (RESV isomers and reference inhibitors) on the enzymatic activity of human recombinant MAO isoforms expressed in baculovirus infected BTI insect cells

Drug	MAO-A (IC_{50})	MAO-B (IC_{50})	Ratio
Clorgyline	$7.27 \times 10^{-3} \pm 1.54^{*}$	59.36 ± 1.48	8165.06
Deprenyl	$72.67 \pm 4.52^{*}$	$30.10 \times 10^{-3} \pm 2.19$	0.000414
Iproniazide	6.68 ± 0.83	8.93 ± 0.44	1.34
c-RESV	$24.65 \pm 0.92^{\#}$	61.29 ± 1.89	2.49
t-RESV	$17.36 \pm 0.65^{\#}$	30.77 ± 0.99	1.77

Results are means ± SEM from five experiments. Level of statistical significance: $^{\#}p < 0.05$ or $^{*}p < 0.01$ versus the corresponding IC_{50} values obtained against MAO-B, as determined by ANOVA/Dunnett's.

It is well known that the "first generation" of antidepressant drugs, the tricyclic antidepressants (tricyclics), introduced in the late 1950s, exhibit in the in vitro studies a fundamental biochemical effect: the non-selective inhibition of NA/5-HT uptake. Since the development of tricyclics, the number of new classes of antidepressant with effects on monoamine uptake used to treat major

depressive disorders has grown dramatically. They mainly include the selective NA and 5-HT uptake inhibitors (see, e.g. [15,17]).

Taking into account the above considerations, in this study we first investigated the potential effects of *c*-RESV and *t*-RESV on [^{3}H]NA/[^{3}H]5-HT uptake.

In our experiments, imipramine concentration-dependently and non-selectively inhibited the uptake of [^{3}H]NA and [^{3}H]5-HT by synaptosomes from rat brain. In contrast, both isomers of RESV, at concentrations within the same range at which these polyphenols have biological effects in in vitro experiments (for a review, see, e.g. [6]), were slight but significantly selective inhibitors of [^{3}H]5-HT uptake against [^{3}H]5-NA uptake ([^{3}H]5-HT uptake selectivity ratios: 1.54 and 2.15, respectively), although less potent and selective than the reference drugs fluoxetine (ratio: 4.02) and citalopram (ratio > 6.33). Furthermore, *t*-RESV was slightly more efficient than *c*-RESV.

On the other hand, the RESV isomers also inhibited the [^{3}H]5-HT uptake by human platelets, *c*-RESV being slight, but significantly less effective than *t*-RESV.

These results indicate that these natural compounds could be potentially useful in the treatment of depression if they exhibited the above-mentioned biochemical activity in vivo. Further clinical studies in humans are required to explore this possibility.

The uptake of [^{3}H]5-HT by synaptosomes from rat brain was less sensitive than the uptake of [^{3}H]5-HT by human platelets to the inhibitory effects of all reference drugs tested. In this connection, it is interesting to note that the results obtained in this study for all reference drugs agree with those previously described using similar experimental conditions (see, e.g. [28,29]).

In contrast, [^{3}H]5-HT uptake by human platelets and synaptosomes from rat brain showed almost equal sensitivity to the inhibitory effects of the RESV isomers. These apparent discrepancies are probably due to the fact that both *c*-RESV and *t*-RESV not only have inhibitory effects on [^{3}H]5-HT uptake by human platelets but also affect the platelet function (e.g., they inhibit the human platelet aggregation induced by a number of aggregatory agents; see [14].

Besides tricyclics, it is well known that other classes of first generation of antidepressant drugs, also introduced in the late 1950s, include the non-selective MAO inhibitors. Recently, the selective MAO-A inhibitors (e.g., moclobemide) have been also developed [15,17]. Thus, we then investigated the possible effects of *c*-RESV and *t*-RESV on the enzymatic activity of human recombinant MAO isoforms.

MAO exist in two isoforms, MAO-A and MAO-B, which share approximately 70% sequence identity on the amino acid levels. These isoforms catalyse the metabolization by oxidation of several amine-containing substrates to yield the corresponding aldehydes, ammonia, and H_2O_2. *p*-Tyramine, which is oxidized to *p*-hydroxyphenylacetaldehyde, is a common substrate for both MAO-A and MAO-B. 5-HT and NA are, however, preferentially oxidized by MAO-A whereas benzylamide and phenylethylamide are preferentially transformed by MAO-B (for reviews, see, e.g. [30–32]).

The production of H_2O_2 catalysed by MAO isoforms can be detected using 10-acetyl-3,7-dihydroxyphenoxazine (Amplex Red reagent), a non-fluorescent, highly sensitive, and stable probe, which reacts with H_2O_2 in the presence of horseradish peroxidase to produce a fluorescent product, resorufin.

In our experiments, both *c*-RESV and *t*-RESV inhibited the fluorescence emitted by the reaction between H_2O_2 and Amplex Red reagent, i.e., the production of H_2O_2 (and, consequently, of resorufin) from *p*-tyramine, using standard commercial isoforms of MAO (human recombinant MAO). These results clearly indicate that the RESV isomers are inhibitors of MAO activity. In this connection, both RESV isomers were slight but significantly selective inhibitors of MAO-A, although less potent and selective than the reference drug clorgyline. In addition, *c*-RESV was slightly less effective than *t*-RESV as inhibitor of MAO-A and MAO-B activity (see Results).

These data partially disagree with those obtained by Zhou et al. [18], who reported that *t*-RESV is a selective inhibitor of MAO-A in rat brain mitochondrial fraction (IC_{50} of 38 μM) and has no effect on MAO-B activity. These discrepancies are probably due to the different experimental conditions used by the above authors (e.g., rat brain mitochondrias as source of enzyme).

Taking into account our results, it is likely that the potential utility of the RESV isomers in the treatment of depression as a result of their inhibitory effects on [^{3}H]5-HT and [^{3}H]NA uptake described above could be amplified apparently by their inhibitory effects on MAO activity.

To summarize, this study shows that the RESV isomers have inhibitory effects on the uptake of [^{3}H]NA and [^{3}H]5-HT by synaptosomes from rat brain, on the uptake of [^{3}H]5-HT by human platelets, and on MAO isoform (MAO-A/MAO-B) activity.

These pharmacological effects caused by *t*-RESV and *c*-RESV were qualitatively similar and quantitatively very close, which suggests that the different spatial conformation of *c*-RESV (versus that of the *trans* isomer) does not seem to modify markedly its interaction with MAO isoforms and with the potential cellular targets implicated in the [^{3}H]NA or [^{3}H]5-HT uptake by synaptosomes from rat brain or in the [^{3}H]5-HT uptake by human platelets.

Bearing in mind these pharmacological properties and assuming that the RESV isomers show similar behaviour in humans in vivo, it can be concluded that these natural phenolic compounds may have interesting therapeutic potential as original chemical models for the design and subsequent development of new drugs (potentially useful for improving the pharmacological treatment of depression) with two important biochemical effects combined in the same chemical structure: NA/5-HT uptake and MAO inhibitory activity.

Acknowledgments

This work was supported in part by grants from the Spanish Ministerio de Ciencia y Tecnología (SAF2002-0245), Almirall-Prodesfarma Laboratories (Pharmacology Award 2003), and the Xunta de Galicia (PGIDIT05BTF20302PR), Spain. Francisco Orallo is especially grateful to Almirall-Prodesfarma Laboratories and the Spanish Pharmacological Society for granting him the 2003 Pharmacology Award. The authors thank Dr. Eugenio Uriarte and Elías Quezada (Department of Organic Chemistry of the University of Santiago de Compostela, Spain) for kindly providing us with *c*-RESV.

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