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Polyphenols as superoxide dismutase modulators and ligands for estrogen receptors

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Abstract

The capacity of estrogen and stilbene derivatives to modulate the activity of superoxide dismutases in relation with their estrogenic properties has been studied. The properties of *trans*-resveratrol (3,5,4'-trihydroxystilbene) and its analogues, 4-hydroxystilbene, 4,4'-dihydroxystilbene, 3,5-dihydroxystilbene, 3,5,4'-trimethoxystilbene and 4,4'-dihydroxy-3,5,3',5'-tetramethylstilbene were compared to 17 β -estradiol and its analogues (2-methoxyestradiol, estrone, 2-hydroxyestradiol and 2-methoxyestrone). Measurement of estrogen receptor- β (ER- β) binding capacity was carried out by a receptor competitor assay associated with fluorescence polarisation detection. The superoxide dismutase (SOD) modulation activity was followed with a spectrophotometric assay using the sequence xanthine/xanthine oxidase-2,3-bis[2-methoxy-4-nitro-sulfo-phenyl]-2*H*-tetrazolium-5-carboxanilide (X/XO-XTT). The structure-activity relationship was different for the two series tested. In the estrogenic series, a compound which does not inhibit SOD, is recognized by the ER- β . In contrast for the stilbenic series both properties are parallel each other.

Keywords Estrogen; Stilbenic derivatives; Resveratrol; Superoxide dismutase inhibitors; Estrogen receptor- β ; Polyphenols

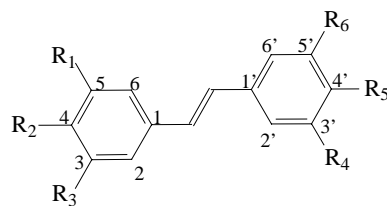
1. Introduction

Resveratrol (3,5,4'-trihydroxy-*trans*-stilbene), a natural phytoalexin found in grapes and wine, shows antioxidant, estrogenic and antiproliferative activities. The anticarcinogenic properties of resveratrol and of some of its derivatives have been demonstrated on different models but the mechanisms by which it acts still remain incompletely understood. Studies on the structural determinants which may be important have been reported: number and position of hydroxyl groups, intramolecular hydrogen bonding, stereoisomery, redox potential [1–5]. Considering the relation between antiestrogenic activity and antiproliferative properties, recent studies have shown that 2-hydroxyestradiol and 2-methoxyestradiol which are es-

trogen derivatives that cannot bind the estrogen receptor, kill human leukaemia cells but not normal lymphocytes [6], and are cell growth inhibitors [7]. One mechanism proposed explains these antiproliferative activities by an inhibition of superoxide dismutase (SOD) by these antiestrogens [6,8,9]. Inhibition of SOD causes accumulation of cellular superoxide radical and, after free radical-mediated damage, leads to apoptosis of the cancer cells. Taking these recent results into account, we were interested in studying the ability of stilbene derivatives (Fig. 1) to modulate the activity of superoxide dismutases in relation with their estrogenic properties. The properties of *trans*-resveratrol were compared to stilbene derivatives which were synthesised as previously reported [1]. Five estrogen derivatives (Fig. 2) were also introduced in this study, among them 17 β -estradiol as a control for estrogen receptor- β (ER- β) binding assays and 2-methoxyestradiol because it cannot bind ER- β and it has been proposed as an inhibitor of SOD [6]. Measurement of estrogen receptor- β (ER- β) binding

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Numbering	R ₁	R ₂	R ₃	R ₄	R ₅	R ₆	Usual Name
S1	-H	-OH	-H	-H	-H	-H	4-hydroxystilbene
S2	-H	-OH	-H	-H	-OH	-H	4,4'-dihydroxystilbene
S3	-OH	-H	-OH	-H	-H	-H	3,5-dihydroxystilbene
S4	-OH	-H	-OH	-H	-OH	-H	3,5,4'-trihydroxystilbene
S5	-OCH ₃	-H	-OCH ₃	-H	-OCH ₃	-H	3,5,4'-trimethoxystilbene
S6	-CH ₃	-OH	-CH ₃	-CH ₃	-OH	-CH ₃	4,4'-dihydroxy-3,5,3',5'-tetramethylstilbene

Fig. 1. Stilbenic compounds studied, S1–S6.

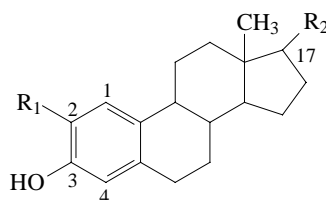
capacity was carried out by a receptor competitor assay associated with fluorescence polarisation detection [10]. The SOD modulation activity has been evaluated with a spectrophotometric assay using the sequence xanthine/xanthine oxidase-2,3-bis[2-methoxy-4-nitro-sulfo-phenyl]-2*H*-tetrazolium-5-carboxanilide (X/XO-XTT) [11].

2. Experimental

2.1. Reagents

Reagents purchased from Sigma–Aldrich (St Quentin Fallavier, France) were sodium carbonate (Na₂CO₃), EDTA (disodium salt dihydrate), XTT sodium salt (2,3-bis[2-methoxy-4-nitro-sulfo-phenyl]-2*H*-tetrazolium-5-car-

boxanilide), xanthine(2,6-dihydroxypurine), xanthine oxidase (EC 1.1.3.22; 0.6 units/mg protein) from buttermilk, Mn-SOD (EC 1.15.1.1; 4400 units/mg protein) and Fe-SOD (EC 1.15.1.1; 2530 units/mg protein) from bovine erythrocytes, 17β-estradiol (E1), estrone (E2), 2-hydroxyestradiol (E3), 2-methoxyestrone (E4), 2-methoxyestradiol (E5) and resveratrol (3,5,4'-trihydroxystilbene/S4). Stilbene derivatives: 4-hydroxystilbene (S1), 4,4'-dihydroxystilbene (S2), 3,5-dihydroxystilbene (S3), 3,5,4'-trimethoxystilbene (S5) were synthesized as previously reported [1]. 4,4'-Dihydroxy-3,5,3',5'-tetramethylstilbene (S6) was synthesized as reported in [12]. All solutions were prepared with water purified by a Milli-Q system (Millipore, Paris). XTT and xanthine were dissolved in a 50 mM sodium carbonate buffer (pH 9.8) at room temperature and at 45 °C, respectively.



Numbering	R ₁	R ₂	Usual Name
E1	-H	-OH	17β-estradiol
E2	-H	=O	estrone
E3	-OH	-OH	2-hydroxyestradiol
E4	-OCH ₃	=O	2-methoxyestrone
E5	-OCH ₃	-OH	2-methoxyestradiol

Fig. 2. Estrogenic compounds studied, E1–E5.

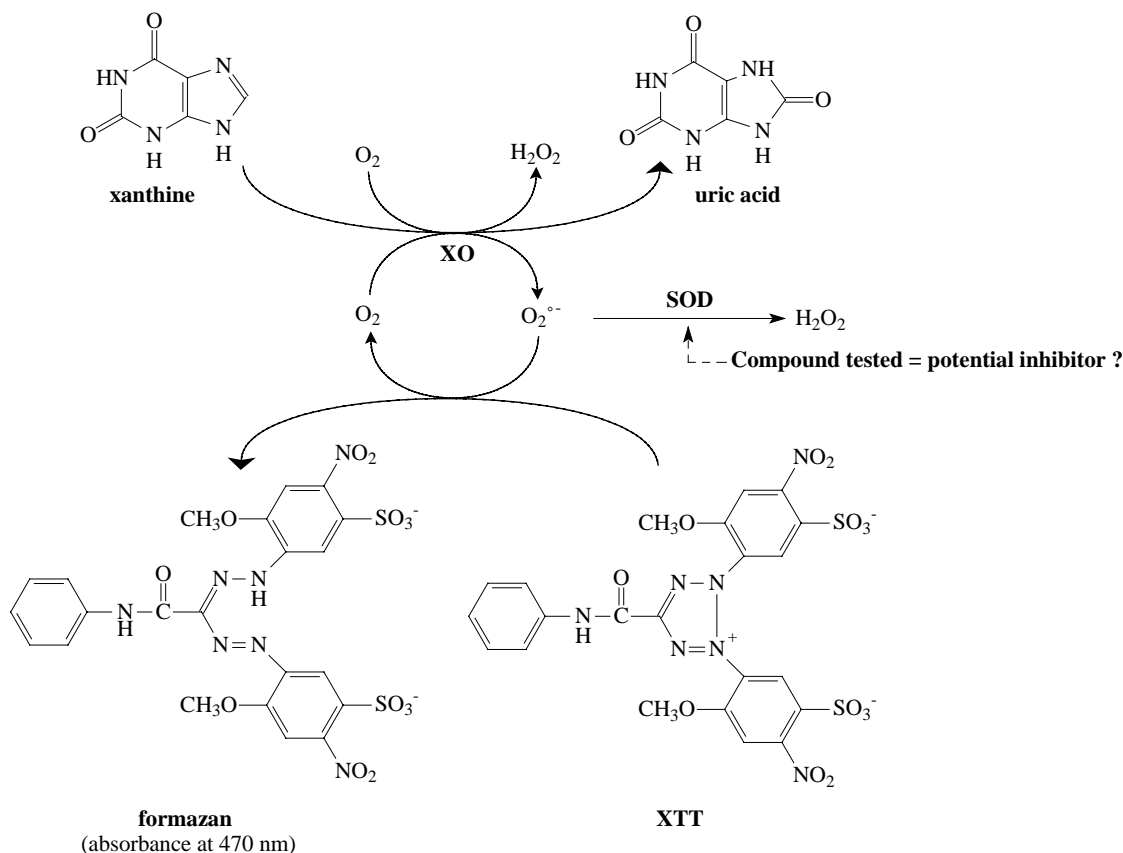
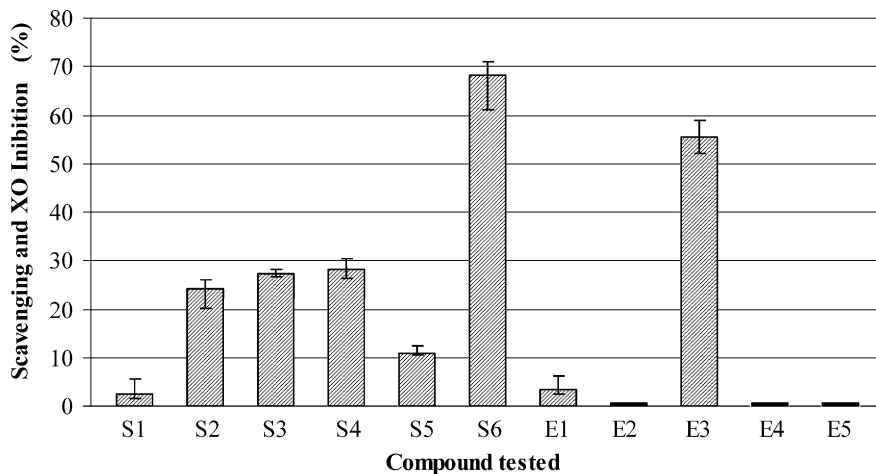


Fig. 3. In vitro SOD assay.

2.2. In vitro SOD assay

The SOD assay using the X/XO-XTT system was carried out as previously reported [11]. The samples tested (final volume 2 ml), distributed on a 24-well microplate (Greiner bio-one, Poitiers, France) were prepared by addition to a 50 mM sodium carbonate buffer (pH 9.8): (i) 67 μ l each of 3 mM EDTA, 0.8 mM XTT, 3 mM xanthine; (ii)

the compound tested at a final concentration in the assay of 1, 10 and 100 μ M; (iii) SOD with a concentration that allowed complete superoxide radical consumption. Instead of SOD, buffer was added to evaluate the XO inhibition by the compound tested (SOD-free control). The reaction was initiated by the addition of XO solution at a concentration of 60 mU ml⁻¹ in the assay. The absorbance change at 470 nm for 30 min was monitored with a microplate reader

Fig. 4. Direct $O_2^{\bullet-}$ scavenging and/or inhibition of XO by the compounds tested (100 μ M).

(BMG Polarstar from BMG Labtechnologies, Champaign sur Marne, France) thermostated at 25 °C. The relative SOD modulation activities (RA) of the compound tested were expressed as:

$$RA = \left[\frac{1 - (c - b)}{a - b} \right] \times 100\%$$

where a is the absorbance without SOD in the presence of the compound tested, b the absorbance with SOD and without the compound tested, c is the absorbance with SOD in the presence of the compound tested.

2.3. *In vitro* β -receptor assay

The affinity of the compounds tested for the human estrogen receptor- β (ER- β) was assayed using the estrogen receptor- β competitor assay distributed by Panvera Co. (Takara Biomedical Europe, Gennevilliers, France) [10]. In this assay ER- β is added to a fluorescent estrogen (Fluormone[®], ES2) ligand to form an ES2/ER- β complex with high fluorescence polarisation. The complex is then added to the estrogen competitor, and the compound tested in microtiter wells. The shift in polarisation after incubation in the dark at 25 °C for 2 h in the presence of the compound tested is used to determine its relative affinity for ER- β . In each well (Falcon, black round microtiter plates, 96 wells) (Becton Dickinson Labware Europe, Le Pont de Claix, France), the concentrations of ER- β , fluormone ES2 and the compound tested were fixed at 20 nM, 2 nM and 20 μ M, respectively. Fluorescence polarisation was monitored with the microplate reader (BMG Polarstar) with 485 nm excitation and 530 nm emission interference filters.

3. Results and discussion

3.1. *In vitro* SOD assay

The superoxide dismutase assay based on the use of the tetrazolium salt XTT was used to evaluate the capacity of estrogen and stilbene derivatives to inhibit or modulate the SOD activity. Fig. 3 presents the principle of the SOD assay which consists in generating the superoxide radical, $O_2^{\bullet-}$, with the xanthine/xanthine oxidase system. The $O_2^{\bullet-}$ radical causes XTT reduction, leading to the formation of reddish water-soluble formazan with an absorbance maximum at 470 nm. In the presence of SOD, the $O_2^{\bullet-}$ radical dismutation reaction ($k \approx 5 \times 10^5 \text{ M}^{-1} \text{ s}^{-1}$) to hydrogen peroxide (H_2O_2) is considerably accelerated ($k \approx 1.6 \times 10^9 \text{ M}^{-1} \text{ s}^{-1}$ at physiological pH). As a consequence of $O_2^{\bullet-}$ consumption by the SOD, the absorbance at 470 nm is reduced in a concentration-dependent way. In a first step, assays without SOD (controls) were carried out to evaluate the capacity of the compounds tested to scavenge the superoxide radical directly and/or to inhibit the xanthine oxidase (Fig. 4). Compounds S6 and E3 led to a strong decrease in absorbance (68 and 55%, respectively) at 100 μ M and may

react directly with $O_2^{\bullet-}$ or inhibit XO. Other compounds tested produced a weaker absorbance decrease between 0 and 28%. These preliminary controls are essential to avoid misinterpretation of the potential activity of the compound tested. It can be noted that E5 (2-methoxyestradiol) does not scavenge the superoxide radical directly and does not inhibit the XO enzyme thus confirming results previously reported by Kachadourian [13]. The antioxidant properties of some stilbenic compounds (S1, S2, S3 and S4) towards $O_2^{\bullet-}$ radicals in a DMSO medium have been previously reported by Privat et al. [1]. This study gave the IC_{50} values which correspond to the stilbene concentration necessary for a 50% decrease of the DMPO-OOH EPR signal intensity according to a control performed without additives. IC_{50} values varied from 820 μ M for 4,4-dihydroxystilbene (S2) to 1680 μ M 3,5-dihydroxystilbene (S3). These concentrations were 8–16 times higher than those used in the X/XO-XTT assay in this work with a maximum concentration of 100 μ M. By using a lower concentration of the compound tested, the scavenging effect is minimised. Moreover, possible interferences which could be observed by direct reduction of XTT (via the direct reaction of the compound tested with the probe) without involvement of superoxide anion, could be suppressed by simple dilution of the sample [14].

In a second step, the critical SOD concentration leading to total superoxide radical consumption was looked for. For fixed concentrations of XO (60 mU ml^{-1}) and XTT (0.8 mM) in the assay, Fig. 5A and B show absorbance versus time for concentrations of Mn-SOD and Fe-SOD between 0 and 10 μ g ml^{-1} . In the absence of SOD in the assay, the absorbance increases linearly during the first 12 min. A small concentration of Mn-SOD (0.1 μ g ml^{-1}) in the assay is sufficient to induce rapid consumption of superoxide. Consequently, compared to the assay without SOD and for a fixed reaction time, a significant decrease of absorbance is observed when the SOD concentration is increased. Increasing the SOD concentration leads to the conditions for absorbance equal to zero which means complete superoxide consumption by the SOD (Fig. 6). Thus, the conditions for a complete superoxide consumption (absorbance at 30 min ≤ 0.004) were obtained: [Mn-SOD] = 2 μ g ml^{-1} and [Fe-SOD] = 8 μ g ml^{-1} . Using these concentrations, the capacity of the natural (E1–E5) and synthetic (S1–S6) estrogens to modulate the SOD activity was evaluated. Fig. 7 presents the absorbance variation in the X/XO-XTT assay versus the concentration of 2-methoxyestradiol (E5) (0–100 μ M). Absorbance was corrected taking into account the direct scavenging activity and/or XO inhibition activity in the control X/XO-XTT assays of the compound tested (Fig. 4). Since E5 does not directly scavenge the $O_2^{\bullet-}$ radical or inhibit the XO enzyme (Fig. 4), the results presented in Fig. 7 confirm the ability of E5 to inhibit SOD. SOD inhibition has recently been reported in different papers pointing out the strong interest of these molecules in anticancer research [4–6,8,9,15]. IC_{50} values represent the concentration that inhibits 50% of the SOD activity and val-

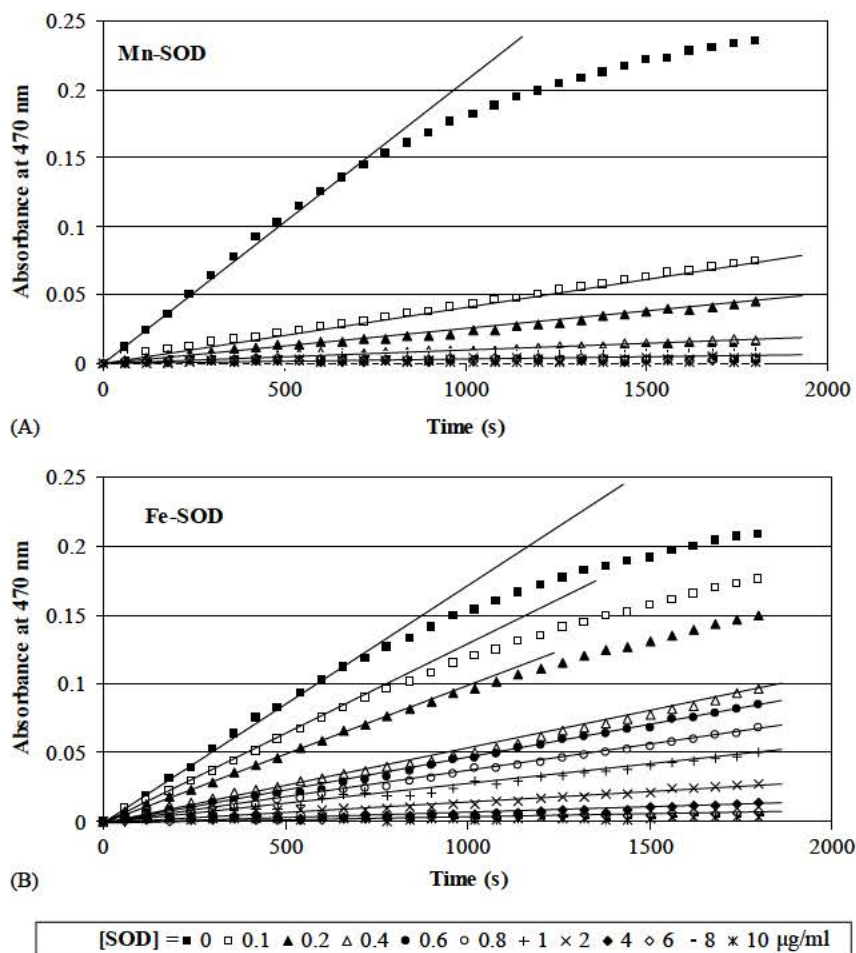


Fig. 5. Absorbance at 470 nm vs. time for various concentrations of SOD.

ues for E5 against Fe-SOD and Mn-SOD are equal to 10 and 20 $\mu\text{g ml}^{-1}$, respectively, in our experimental conditions.

Fig. 8 compares the relative activities of the compounds tested in inhibiting Mn-SOD (Fig. 8A) and Fe-SOD

(Fig. 8B) as a function of the concentration (1, 10 and 100 μM). In the stilbene series, S3 and S5 do not inhibit SOD while S1, S2 and S4 are strong inhibitors. In the estrogen series, the SOD inhibiting and/or modulating activities

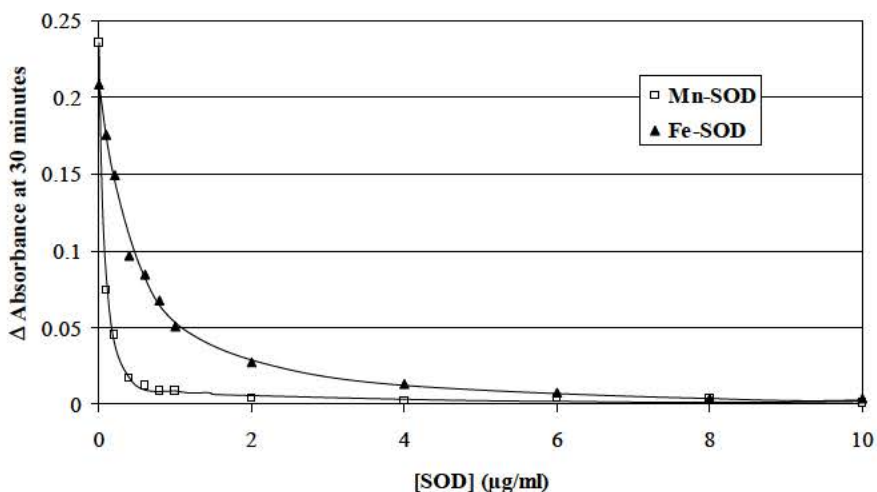


Fig. 6. $\text{O}_2^{\bullet-}$ dismutation by SODs.

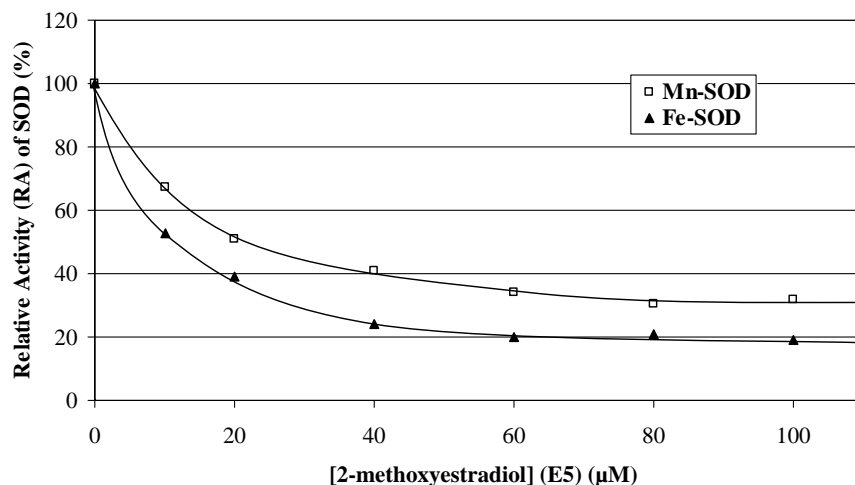


Fig. 7. Inhibition of Mn- and Fe-SOD by different concentrations of 2-methoxyestradiol (E5).

are less contrasted. Whereas E1 and E2 present a very weak modulating activity, E3, E4 and E5 decreased the SOD activities (to 55–25%) at 100 µM.

It should be emphasised that a larger amount of Fe-SOD than Mn-SOD was necessary to dismutate the total amount

of $O_2^{\bullet-}$ generated by the X/XO system in our experimental conditions. This lower activity of Fe-SOD compared to Mn-SOD leads to the appearance of a higher activity of the compounds against Fe-SOD at the same concentration tested.

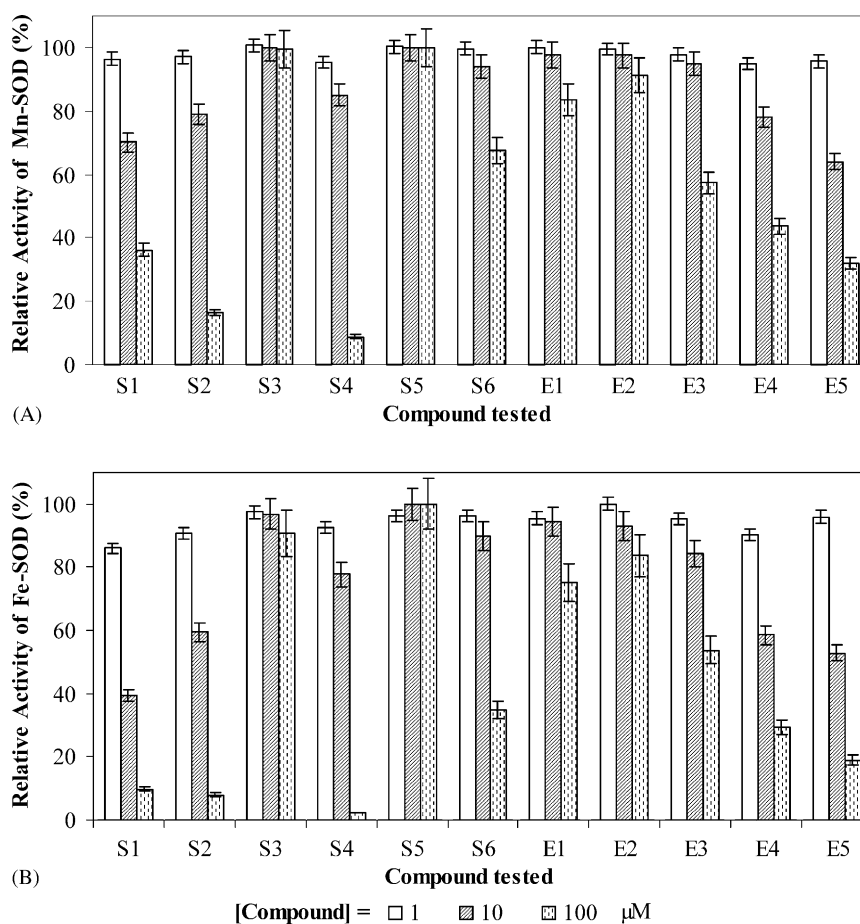


Fig. 8. Relative activities of SODs vs. the compound concentration.

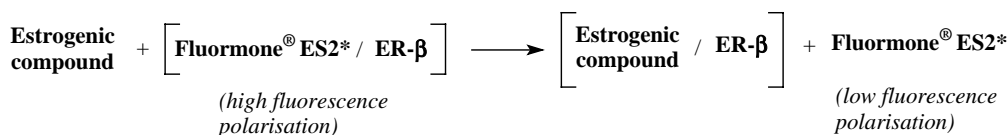


Fig. 9. In vitro estrogenic receptor- β assay (ER- β).

3.2. In vitro β receptor assay

Fig. 9 illustrates the competitive assay used to assess the affinity of the series tested (E1–E5, S1–S6) for the human estrogen receptor- β (ER- β). The concentration that results in a half-maximum shift in the polarisation gives the IC_{50} of the compound tested. The IC_{50} value corresponds to the concentration of competitor needed to displace half of the bound ligand. Fig. 10 presents the competition binding assay curve for the 17 β -estradiol (E1) leading to an IC_{50} of about 20 nM. This result is in agreement with published data [10]. To compare the binding capacity of the compounds to ER- β , a fixed concentration was used (1.0×10^{-5} M). The compounds S3, S5, S6, E3, E4 and E5 appear as non- or very weak competitors of fluormone[®] ES2 while S1, S2, S4, E1 and E2 are clearly recognized by ER- β (Fig. 11).

Overall examination of the results shows that the two series studied remain distinct. In the estrogen series, when a compound does not inhibit SOD, it is recognized by ER- β . In this way, E3 and E4 behave like 2-methoxyestradiol (E5). In the stilbene series, when a compound presents a SOD-inhibiting-like activity, it is also recognized by ER- β and when it has no SOD inhibiting activity, it is not recognized. The exception is S6 for which a strong $O_2^{\bullet-}$ scavenging activity and/or an inhibition of the XO enzyme was observed in the control experiment. Inhibition of SOD activity and ER- β recognition by stilbene compounds seems linked to the presence of the OH group on the R_2 or R_5 posi-

tions (S1, S2, S4). In the estrogen series, the R_2 substituent is not preponderant for the SOD inhibiting activity or for ER- β recognition. The R_1 substituent, on the contrary, plays an important role: the substitution of the H atom by OH or OCH_3 reverses the activities. These structural considerations are in agreement with previously reported results [5–9,16]. Our results are consistent with the previous study of Huang et al. [6]. First of all, the order and magnitude of SOD inhibition by the estrogen derivatives is respected. Secondly, we confirm that 2-methoxyestradiol (E5) has no effect on xanthine oxidase (inhibition and/or $O_2^{\bullet-}$ scavenging) and that this compound inhibits SOD activity or, in all cases, modulates the enzyme activity. Chemical modifications at the 2-carbon substituent (R_1 : OH, OCH_3) of the estrogen derivatives are essential for SOD inhibition, which is the case for E5, E3 and E4. In contrast, 17 β -estradiol (E1) and estrone (E2), which lack the carbon-2 modification, showed minimal activity against SOD. Interestingly, a similar effect is found in the stilbene series: 4-hydroxystilbene (S1), 4,4'-dihydroxystilbene (S2) and 3,5,4'-trihydroxystilbene (S4) which have an OH group at the 4 and/or 4'-carbon (R_2 or R_5 substituent) present the most effective inhibition of SOD. 3,5-Dihydroxystilbene (S3), which lacks the 4 and/or 4'-carbon OH group, does not present a modulating effect on the SOD activity. An exception is found with 4,4'-dihydroxy-3,5,3',5'-tetramethylstilbene (S6), though it has the 4 and/or 4'-carbon OH group, it is not a SOD inhibitor. This might be due to the steric effects of the methyl groups.

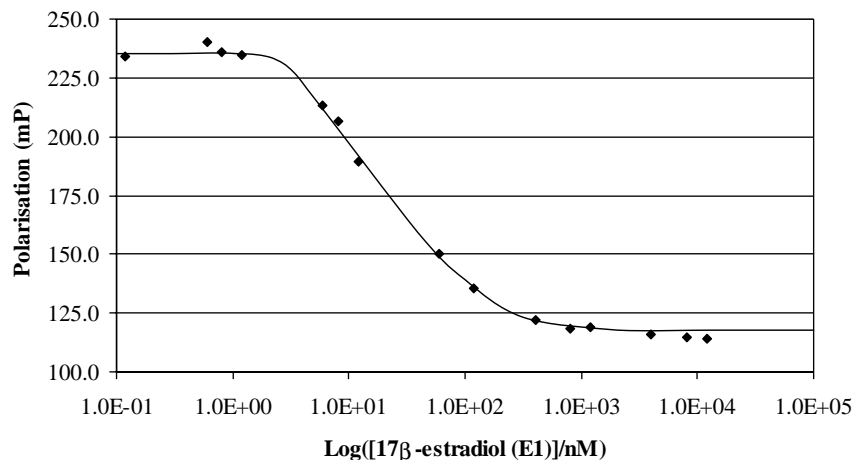


Fig. 10. ER- β binding assay with 17 β -estradiol (E1).

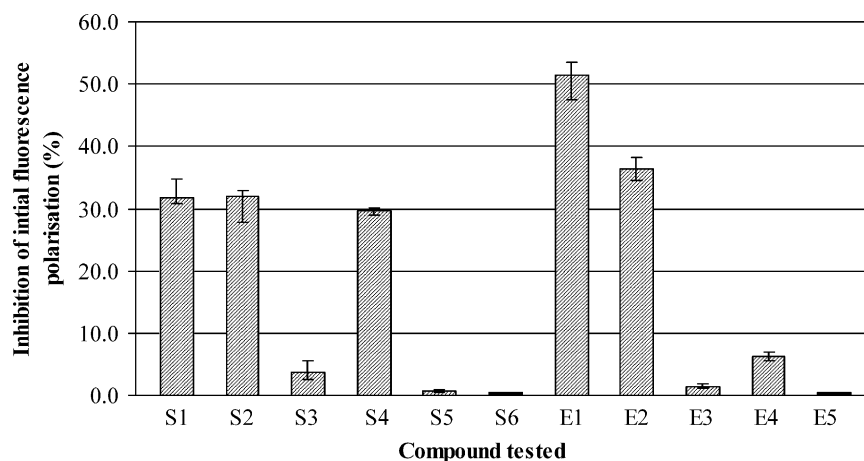


Fig. 11. ER- β binding assay with fixed concentration of competitor (1×10^{-5} M).

The preponderant effect of the hydroxyl group in the 4' position in stilbene derivatives was underlined by Stivala et al. [5] who showed that the presence of 4'-OH together with a *trans*-configuration (4'-hydroxylstyryl moiety) was absolutely required for the inhibition of fibrosarcoma cell proliferation. In this case, the active $O_2^{\bullet-}$ production and low SOD activity (due to inhibition by the compound with hydroxyl group in 4' position) may render the malignant cells highly dependent on SOD for survival and hence sensitive to SOD inhibition [5].

Inhibition of superoxide dismutases by the 2-methoxyestradiol initially described [6] has been confirmed twice [8,16]. Another study [17] has found a selective inhibition of Fe- versus Cu/Zn-SOD by 2,3-dihydroxybenzoic acid derivatives. Although this finding was questioned in one study [13], our observations confirm that 2-methoxyestradiol inhibits SOD activity and that some stilbene derivatives can effectively modulate superoxide dismutase activity. Moreover, in the stilbene series, the structure-activity relations established for the modulation of SOD activity appear to be applicable to ER- β recognition. Stilbene compounds which have a hydroxyl group at the 4 and/or 4'-carbon are recognized by ER- β . 3,5-Dihydroxystilbene (S3), which lacks this 4 and/or 4' substituent, is clearly not recognized by ER- β . As for the modulation of the SOD activity, an exception is found with the 4,4'-dihydroxy-3,5,3',5'-tetramethylstilbene (S6), likely because of steric effects by the methyl groups. In the estrogen series, our results confirm that 2-methoxyestradiol (E5) and 2-hydroxyestradiol (E3) have little affinity for ER- β [7,9]. Recognition by the estrogen receptor seems linked to the presence of a hydrogen atom on the 2-carbon (R_1 substituent) coupled with the hydroxyl group on the 3-carbon. This observation is respected in the stilbene series: the recognition is effective when a hydroxyl group is located at the 4 and/or 4'-carbon and when a hydrogen atom is present on the vicinal carbon.

In conclusion, when compounds in the estrogen series are inhibitors of SOD (or not), they are not (or are) recognized by ER- β ; in the stilbene series both properties exist together.

Acknowledgements

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