Neuroprotective potential of aromatic alcohols against oxidative cell death

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Received 11 July 1997

Abstract Estrogens can protect neurons against oxidative stress-induced cell death due to their antioxidant potential. Here, we report that other aromatic alcohols with intact phenolic groups and different phenol derivatives can also protect neurons against oxidative cell death induced by glutamate and hydrogen peroxide in vitro. This neuroprotective activity was independent of the time the compound was added before the toxin. Methylation of the phenolic hydroxyl group led to a decrease or loss in neuroprotection. Moreover, the tested compounds directly inhibited peroxidation reactions, suggesting that neuroprotection is mediated by their antioxidant properties.

Key words: Aromatic alcohol; Glutamate; Oxidative stress; Neurodegeneration; Lipid peroxidation; Antioxidant

1. Introduction

Oxidative stress describes the imbalance between free radical generation and various enzymatic and non-enzymatic antioxidant defense systems and may contribute to a variety of pathological conditions including atherosclerosis and several neurodegenerative disorders. Brain cells are at a particular risk from damage caused by oxidative stress (for review [1–5]).

The excitatory amino acid glutamate may be cytotoxic to neurons [6,7]. It has been shown in various experimental paradigms that glutamate can induce the generation of several reactive oxygen species in neurons indicating a general role of free radicals in glutamate toxicity (for review [3]). In the clonal murine hippocampal cell line HT22, glutamate induces an increase in the level of intracellular H_2O_2 and, ultimately, oxidative cell death in a glutamate receptor-independent manner [8]. H_2O_2 is the precursor of highly reactive hydroxyl radicals which can attack unsaturated side chains of the membrane's lipid molecules, leading to peroxidations (for review [4,9]).

In the search for potential antioxidants for neurons, we used glutamate- and H_2O_2-induced cell death in HT22 cells and primary cortical neurons from rat as toxicity paradigms. So far, several antioxidants have been described to prevent oxidative stress-induced neuronal cell death including *N*-acetyl-5-methoxytryptamine (melatonin) [10] and 17-β-estradiol [11,12]. Recently, we described the presence of the free C3 hydroxyl group of the estrogen molecule's A-ring, which represents a phenolic group, as the only structural prerequisite necessary for the antioxidative and neuroprotective effect of this aromatic alcohol [13]. Using liver microsomes it has been previously shown that natural phenolic compounds may potentially prevent peroxidative damage [14–16].

To investigate whether the antioxidant activity of phenolic groups as present in estrogen does also afford neuroprotective abilities to other molecules, we studied a whole panel of different aromatic alcohols and their derivatives (see Fig. 1) for their activity to prevent oxidative stress-induced cell death and lipid peroxidation in neuronal cells.

2. Materials and methods

2.1. Cell culture and chemicals

The cell line HT22 is a subclone of the HT4 hippocampal cell line, which is sensitive to glutamate [8]. It was a kind gift from Dr. Pamela Maher (The Scripps Research Institute, San Diego, CA) and was cultured as described [8,10]. Rat primary neurons were established from E17 embryonic cerebral cortex and cultured as reported [13]. All media and sera were obtained from Gibco/Life Technologies (Eggenstein, Germany). All chemicals were from Sigma (Deisenhofen, Germany), unless otherwise stated, and were of the highest grade available. 4-Dodecylphenol was administered as a mixture of alkyl chain length isomers, 5-hydroxytryptamine as creatinine sulfate complex. Amines were used as the corresponding hydrochlorides. 5-Hydroxytryptamine, *N*-methyl-5-hydroxytryptamine, 5-hydroxyindole, 4-methoxyphenol and 2-hydroxybenzoic acid were dissolved in water and all other aromatic compounds were tested in ethanol.

2.2. Cytotoxicity and viability assays

Cell viability was determined by two different assays. For microscopical examination and to determine the fraction of dead cells in the culture, cells were stained with propidium iodide (PI; 5 μg/ml PI for 5 min) as described [13] and viewed using fluorescence microscopy. In addition, a modified 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) assay was employed exactly as previously reported [10,11,13]. For the pretreatment of the cells with aromatic alcohols, compounds were added to the cultures 3 h before the toxin. After additional 20 h, MTT assays were performed. A maximal final concentration of 2% ethanol (v/v) did not affect MTT conversion or cell survival in these short time assays. Moreover, no interference of the aromatic compounds with the MTT could be detected. Whenever a compound used decreased the cellular survival too much ($< 50\%$ cell survival) as 17-β-estradiol, 4-dodecylphenol, 4,4′-biphenol at a concentration of 200 μM, any potential protective activities could not be determined. For statistical analysis unpaired Student's *t*-tests were performed.

2.3. Tissue homogenization and preparation of the cell membrane fraction

Adult pig brain tissue was homogenized and membrane fractions were prepared exactly as described [17]. The membrane-enriched fraction was stored in aliquots frozen at −30°C.

2.4. Measurement of in vitro lipid peroxidation by a single photon counting/chemiluminescence assay and by the detection of thiobarbituric acid-reactive substances (TBARS)

The single photon counting/chemiluminescence assay was performed as previously described [17] with only minor modifications using a photon counting apparatus (Luminomat, Berthold). Samples of pig brain cell membranes (0.1 mg protein/ml), 100 μM ascorbic acid and the substance to be tested for the potential peroxidation

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PII S0014-5793(97)00961-7

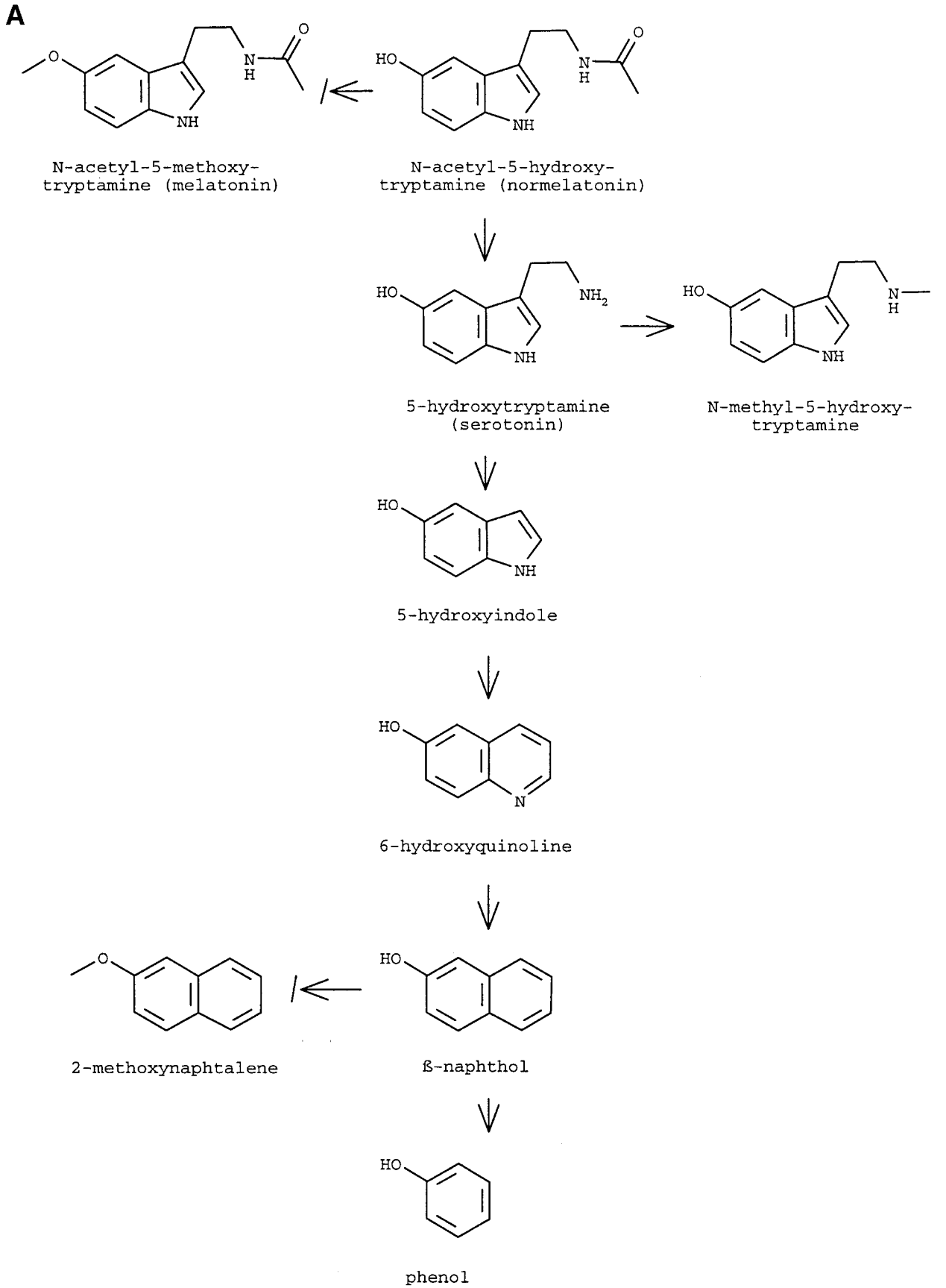

Fig. 1. Molecule structures of different aromatic alcohols and derivatives. The different compounds that were tested for potential neuroprotective activity are depicted. The arrows in these figures do not indicate chemical reactions.

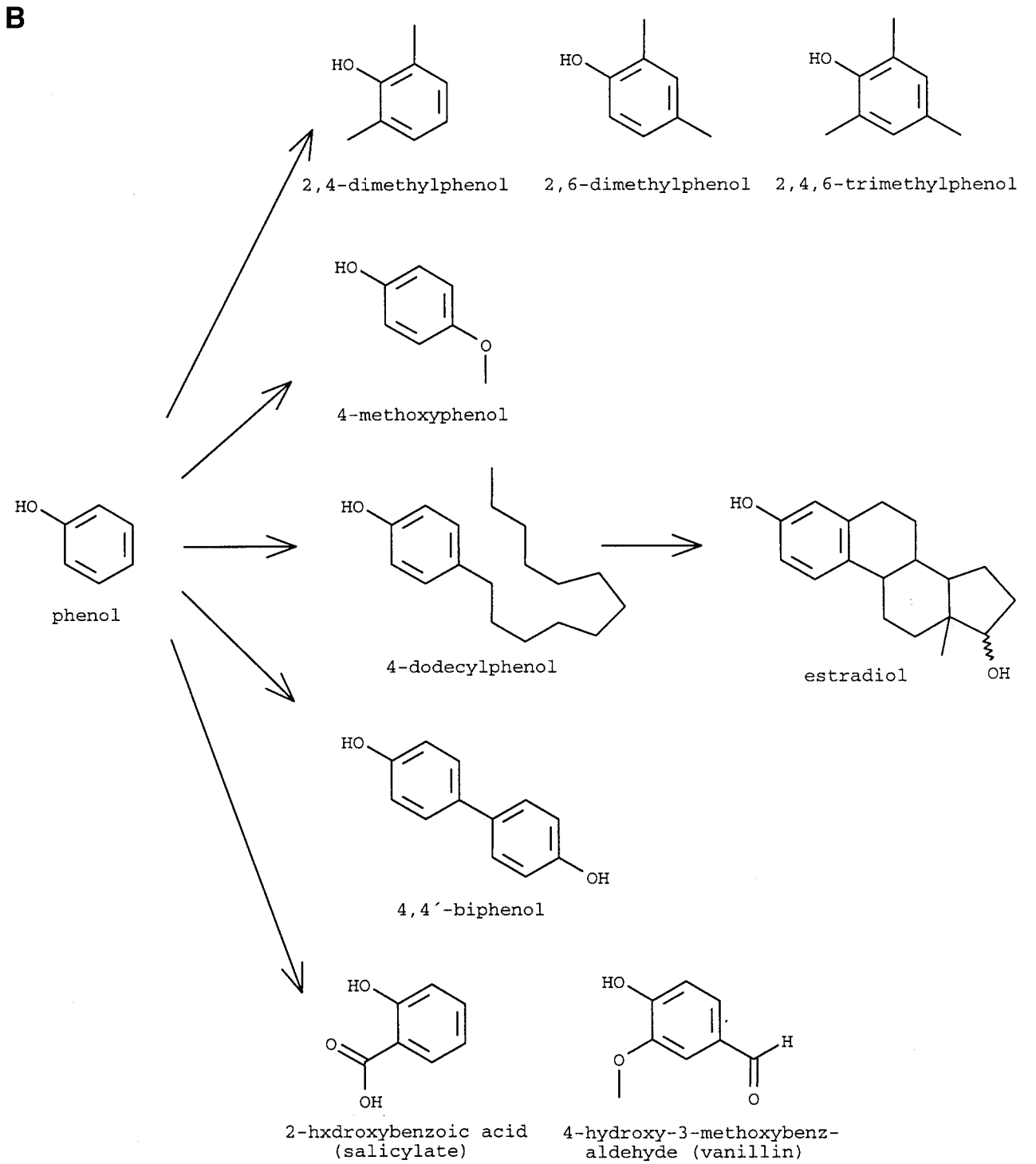

Fig. 1 (continued).

inhibiting/antioxidant effect (in 10 μl of ethanol) were combined in the cuvette to start the spontaneous chemiluminescence reaction. The signals were recorded and the intensity after 1 h calculated as impulses/second (I/s), exactly as reported [17].

Measurement of malondialdehyde (MDA) was performed, as described, with only minor modifications [18]. Separation of the TBARS was carried out using reversed-phase high-performance liquid chromatography (RP-HPLC; LiChrospher 100 RP-18; 5 μm; LichroCART 125-4; Merck). The mobile phase consisted of methanol/0.05 M potassium phosphate buffer (40:60, v/v; pH 6.8) applying a flow rate of 1.5 ml/min. For the calibration curve, MDA was obtained by acid hydrolysis of 1,1,3,3-tetramethoxypropane. The MDA–TBA complex was monitored by fluorescence detection with excitation at 515 nm and emission at 553 nm.

3. Results

In Fig. 1, the chemical structures of the various aromatic alcohols, their derivatives and their precursor (phenol), which were tested for potential neuroprotective activities, are shown.

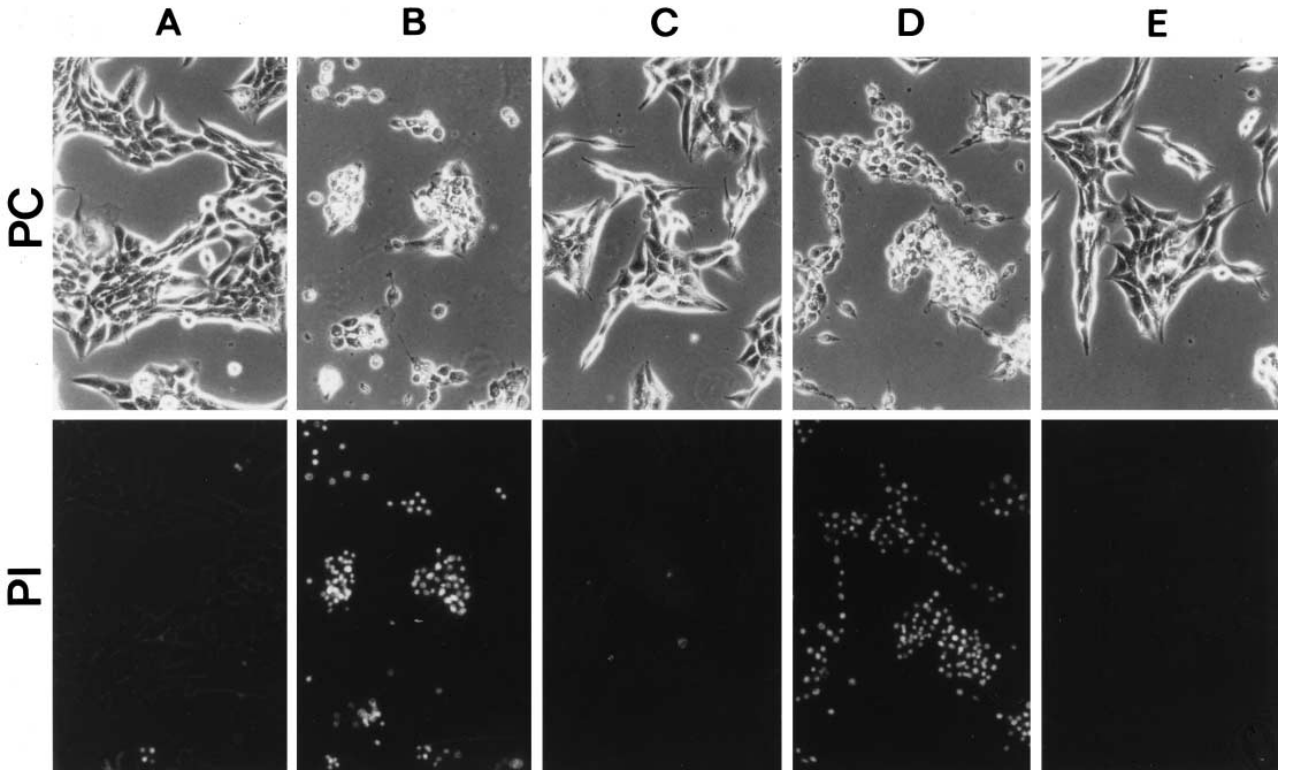

Fig. 2. Protection against glutamate-induced HT22 cell death by β-naphthol and 4-dodecylphenol. HT22 cells were pretreated for 3 h with 200 μM β-naphthol (C), 200 μM 2-methoxynaphthalene (D), 20 μM 4-dodecylphenol (E), or left untreated (B) before an oxidative challenge with 20 mM glutamate. After 24 h, cultures were stained with the vital fluorecent dye propidium iodide (PI) and then viewed using phase contrast (PC) and fluorescence microscopy. Magnification was 100-fold.

In a first set of experiments, the effect of a selection of aromatic alcohols and their derivatives on the survival of glutamate-treated HT22 cells was investigated. After a 24-h incubation with 20 mM glutamate, the fraction of dead (PI-stained) cells was highly increased (Fig. 2B). After a pretreatment of the cultures with different aromatic alcohols containing an intact hydroxyl group, but otherwise considerable structural differences, almost no signs of cellular damage or cell death were observed, as shown for β-naphthol (Fig. 2C) and 4-dodecylphenol (Fig. 2E). In contrast, derivatives with a methylated phenolic hydroxyl group, such as 2-methoxynaphthalene did not prevent glutamate-induced HT22 cell death (Fig. 2D).

In a second step, the different compounds shown in Fig. 1 were investigated within the same toxicity paradigm, but employing the MTT test for quantification of survival. In Table 1, these data are summarized: 4-dodecylphenol and 4,4′-biphenol were highly protective against glutamate at a concentration as low as 5 μM. The different indole derivatives had to be used at a concentration of 20 μM in order to afford significant protection. While β-naphthol was protective against glutamate toxicity also at 20 μM, its ether-modified derivative, 2-methoxynaphthalene, did not increase cellular survival. Salicylate (2-hydroxybenzoic acid) and vanillin (4-hydroxy-3-methoxybenzaldehyde) were not protective (Table 1A). A similar pattern of protective activity of the various compounds could be detected using 250 μM H_2O_2 as oxidative challenge for HT22 cells (data not shown). Consistently, after pretreatment of primary cortical neurons with *N*-acetyl-5-hydroxytryptamine (normelatonin), 5-hydroxytryptamine (serotonin), 5-hydroxyindole, 4,4′-biphenol and as a control with 17-β-estradiol, cells were also significantly protected against oxidative cell death induced by 250 μM H_2O_2 (data not shown). Interestingly, the protective activity was independent from the time the various aromatic compounds were added before the toxin. Addition of the aromatic alcohols at 22, 19, 16, 4, 3, 2, or 1 h before the toxin or together with the toxin resulted in an identical neuroprotective effect. In contrast, addition of the potential protective compounds after toxin addition led to a loss of the protective effect (data not shown).

Finally, two cell-free lipid peroxidation assays were performed in order (1) to determine the potential of selected aromatic alcohols to prevent the direct peroxidation reaction of lipids and (2) to prove a direct antioxidative effect at concentrations similar to those used in the cell assays (Table 1A and Fig. 2). With both assays, we found that at 100 μM, normelatonin and β-naphthol prevented lipid peroxidation reactions to a similar extent like 17-β-estradiol while melatonin and 2-methoxynaphthalene were almost inactive (Table 1B). We, therefore, concluded that compounds that were neuroprotective in the cellular models of oxidative stress-induced neuronal cell death can act as direct inhibitors of lipid peroxidation.

4. Discussion

Different aromatic alcohols can prevent oxidative cell death induced by glutamate and H_2O_2 in clonal hippocampal HT22 cells and in primary cortical neurons. The phenolic compounds 4-dodecylphenol, 4,4′-biphenol, 5-hydroxyindole, se-

Table 1
Protection of clonal hippocampal HT22 cells against glutamate-induced oxidative cell death (A) and inhibition of lipid peroxidation (B) by aromatic alcohols

A. Protection of HT22 cells by aromatic alcohols (% viability ± SEM)			
Control	100		
Glutamate (20 mM) alone	2 ± 4		
Glutamate (20 mM) treatment after 3 h pre-incubation with	5 μM	20 μM	200 μM
N-Acetyl-5-hydroxytryptamine	9 ± 3*	18 ± 6**	89 ± 5**
N-Acetyl-5-methoxytryptamine	5 ± 3	9 ± 5	25 ± 7**
5-Hydroxytryptamine	8 ± 6	28 ± 4**	72 ± 6**
N-Methyl-5-hydroxytryptamine	6 ± 5	15 ± 6*	79 ± 8**
5-Hydroxyindole	14 ± 7*	80 ± 5**	95 ± 4**
6-Hydroxyquinoline	4 ± 3	22 ± 7**	90 ± 5**
β-Naphthol	3 ± 3	42 ± 10**	88 ± 10**
2-Methoxynaphthalene	4 ± 4	4 ± 5	5 ± 3
Phenol	2 ± 3	6 ± 4	60 ± 6**
2,4-Dimethylphenol	3 ± 2	66 ± 6**	59 ± 5**
2,6-Dimethylphenol	6 ± 4	53 ± 7**	66 ± 5**
2,4,6-Trimethylphenol	11 ± 6*	69 ± 8**	64 ± 4**
4-Methoxyphenol	7 ± 4	28 ± 8**	84 ± 5**
4-Dodecylphenol	58 ± 14**	89 ± 7**	n.d.
17-β-Estradiol	30 ± 8**	85 ± 10**	n.d.
4,4′-Biphenol	69 ± 14**	101 ± 8**	n.d.
2-Hydroxybenzoic acid	1 ± 3	6 ± 2	10 ± 7
4-Hydroxy-3-methoxybenzaldehyde	4 ± 3	10 ± 5*	22 ± 10*

B. Lipid peroxidation (in % ± SEM) Control						
	3 μM		30 μM		100 μM	
N-Acetyl-5-hydroxytryptamine	95 ± 4	(95 ± 9)	82 ± 9	(75 ± 10)*	31 ± 7**	(41 ± 12)**
N-Acetyl-5-methoxytryptamine	92 ± 5	(97 ± 9)	96 ± 5	(90 ± 14)	89 ± 4	(82 ± 11)
β-Naphthol	73 ± 3**	(78 ± 11)	4 ± 2**	(14 ± 9)**	0 ± 0**	(7 ± 5)**
2-Methoxynaphthalene	95 ± 7	(95 ± 7)	87 ± 5	(85 ± 9)	80 ± 4	(81 ± 11)
17-β-Estradiol	46 ± 13*	(45 ± 10)**	1 ± 1**	(4 ± 2)**	6 ± 3**	(8 ± 2)**

A: HT22 cells were pretreated with increasing concentrations of the various aromatic alcohols; glutamate was added and MTT tests performed. All results were expressed as percent MTT reduction in toxin-/aromatic alcohol-treated cultures compared to control cultures treated with aromatic alcohols alone and were normalized to control values (no addition of toxin or aromatic alcohol) as 100%. The data are presented as means ± SEM of one representative quadruplicate determination.
B: Pig brain membrane homogenates were prepared and lipid peroxidation reactions detected. Chemiluminescence of the single photon counting chemiluminescence assay is presented as relative light units (mean of 5 independent experiments) compared to the control after subtraction of the background luminescence (in %). The TBARS data are presented in paranthesis. They were calculated from 5 independent experiments each and compared to the control values after subtraction of the baseline.
The P values of $^{*}P < 0.05$ and $^{**}P < 0.01$ were considered as significant; n.d. = not determined.

rotonin, *N*-methyl-5-hydroxytryptamine and normelatonin were as effective as 17-β-estradiol with respect to maximal neuroprotection at comparable concentrations. 6-Hydoxyquinoline, β-naphthol and different methylphenols can protect HT22 cells in the toxicity paradigms used, as well. Common to all the neuroprotective compounds is the presence of a hydroxyl group on the mesomeric ring system of the molecule. Apart from this feature, the structure of the neuroprotective compounds is considerably different.

Recently, we described a neuroprotective activity against glutamate- and H_2O_2-induced oxidative HT22 cell death for different antioxidants, including the phenolic molecule estrogen [11,13]. While estrogens are highly neuroprotective at a micromolar concentration range, melatonin has to be used at much higher concentrations in order to protect HT22 cells (Table 1A) [10]. The poor antioxidant capacity of melatonin in vitro was already demonstrated and discussed elsewhere [19]. Using the aromatic alcohols normelatonin, serotonin, and also 5-hydroxyindole with an intact phenolic hydroxyl group, we now show a higher protective potential for these compounds compared to melatonin. Consistently, while β-naphthol with an intact phenolic group has some protective potential, its methylether counterpart 2-methoxynaphthalene lacks these protective properties.

Lipophilicity may be an enhancer of neuroprotective action because, for example, the transition from mere phenol to 4-dodecylphenol results in a significant increase in lipophilicity of the molecule and also in a significantly increased neuroprotective activity. Interestingly, also 2,4-dimethylphenol and 2,4,6-trimethylphenol can act as neuroprotectants demonstrating that the substitution of one or both free ortho-positions at the aromatic ring does not necessarily inhibit neuroprotective activity.

Antioxidative aromatic alcohols, such as estrogens may donate hydrogen atoms from their phenolic hydroxyl groups to hydroxyl radicals or lipid peroxyradicals (for review [4,9]). This mode of action may also apply to the compounds tested in this study. Because the neuroprotective aromatic alcohols used can directly prevent lipid peroxidation, very likely it is indeed this peroxidation inhibiting potential of the aromatic alcohols that can prevent oxidative neuronal death induced by glutamate and H_2O_2.

The clinical importance and applicability of antioxidant neuroprotection is documented by recent reports that the

treatment of AD patients with vitamin E slows the progression of the disease [20]. In conclusion, our data show for the first time the antioxidant potential of various aromatic alcohols in disease-related paradigms of oxidative toxicity employing cultures of clonal neuronal cells and primary neurons. Moreover, these data further support and largely extend our previous findings that the structural prerequisite of a potent neuroprotective activity of steroids is an intact hydroxyl group on a mesomeric system [13]. Therefore, our data may help in the design of antioxidative and neuroprotective drugs in the future.

Acknowledgements: The authors wish to thank Dr. Frank Lezoualc'h for helpful comments on the manuscript. This work was supported in part by a grant from the 'Wilhelm-Woort-Stiftung für Alternsforschung' to Dr. Christian Behl.

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