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New structures able to prevent the inhibition by hydroxyl radicals of glutamate transport in cultured astrocytes

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Abstract – 4,5,6,7-Tetrahydro-benzothiophen-7-ylamines, 4,5,6,7-tetrahydro-benzothiophen-4-ylamines, and 5,6-dihydro-4*H*-thieno[2,3-*b*] thiopyran-4-ylamines were designed, synthesized, and tested as $OH^{\bullet}$ radical scavengers. Most of them displayed chemical scavenging properties better than or in the same range as salicylic acid. Moreover, some compounds were able to protect in vitro the astroglial glutamate transporters against inhibitory action of radicals promoted by xanthine/xanthine oxidase. Thus, such compounds might be useful for lowering the large amounts of excitotoxic glutamate liberated during acute CNS diseases: they might protect the glutamate reuptake in astrocytes from the inhibitory action due to radicals co-liberated with glutamate. © Elsevier, Paris

free radicals / glutamate transporters / astrocytes / neuroprotection

1. Introduction

Large excitotoxic amounts of glutamate are liberated in the extracellular areas of the central nervous system (CNS) after hypoxia, ischemia or trauma [1]. Following this liberation the stimulation of N-methyl-D-aspartate (NMDA) receptors by glutamate induces a large influx of Ca^{2+} inside nervous cells which disturbs both metabolic and osmotic equilibria causing acute or delayed neuronal death [2–4]. The neuronal death in turn is an additional source of glutamate which goes to spread the primary lesions [5]. It is thus critical to control the extracellular concentrations of glutamate after various acute CNS diseases.

In physiological conditions, astroglial glutamate transporters play apparently a major role in clearance of the neurotransmitter [6]. Consistently the reuptake of extracellular glutamate in astrocytes protects against neurodegeneration due to excitotoxicity [7]. Astroglial cells are thus supposed to play a pivotal role in glutamate homeostasis and so far to participate to neuronal protection. After trauma however, it was shown that free radicals formed from oxygen are liberated simultaneously with glutamate [8–10]. Various endogenous mechanisms are known to produce $O_2^{-\bullet}$ and H_2O_2 which can be easily transformed into the $OH^{\bullet}$ radical by a Fe-catalysis according to the Haber–Weiss reaction [11–13]. The most reactive species $OH^{\bullet}$, among numerous deleterious oxidative actions, is able to oxidize astroglial transporters which are thus inactivated [14, 15]. At this time the astroglial reuptake is inhibited. Accordingly, the sustained high concentrations of extracellular glutamate spreading neuronal lesions may be explained both by glutamate release and by inhibition of glutamate reuptake in astrocytes.

As yet, a well-known strategy to protect neurons after a trauma is the competitive or non-competitive blockade of the NMDA receptors. Actually, we have obtained good experimental results by means of non-competitive NMDA antagonists able to block the Ca^{2+} influx in neurons like 1-[1-(2-thienyl)cyclohexyl]piperidine (TCP), derivatives, and analogues [16–20]. Nevertheless, this effective strategy might be reinforced by the contribution of hydroxyl radical scavengers to the protection

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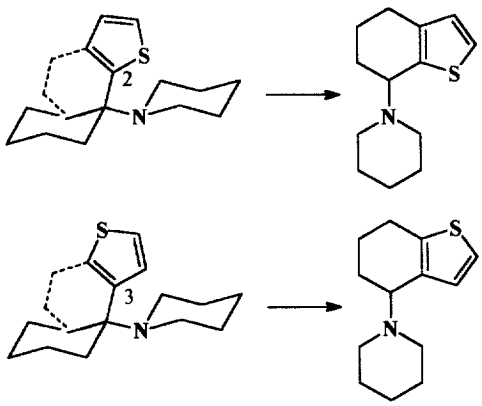

Figure 1.

of glutamate transporters in astrocytes. This would give a more flexible dual treatment and a likely reduction of the amount of non-competitive NMDA antagonists administered, and thus a decrease of their side effects.

With this aim we have modelized new molecules using a strategy based on the following needs: (i) keep the overall physical properties of TCP because it passes easily the blood brain barrier, (ii) keep a thiophene ring known to scavenge radicals [21], (iii) prevent new molecules to bind to the PCP receptor, a property achieved by rigid structures in the arylcyclohexylamines series [22, 23], (iiii) generate molecules with HOMO orbitals energies close to the $OH^{\bullet}$ SOMO orbital energy to obtain specific scavenging properties [24]. By means of the molecular modeling software ChemX® (Chemical Design) and the included semi-empirical molecular orbital package MOPAC 6.0 (QCPE) we have obtained two structures globally in agreement with fixed prerequisites: 1-(4,5,6,7-tetrahydro-1-benzothiophen-4-yl or -7-yl)piperidine (see *figure 1*). Then, we have modulated these two models to obtain several derivatives and analogues. The new compounds were tested for their ability to scavenge radicals and to protect the glutamate transporters in cultured astrocytes against radicals produced by the xanthine/xanthine oxidase (X/XO) mixture [15].

2. Chemistry

Three series of compounds (see *figure 2*) were prepared after the models chosen (see *figure 1*). The first was obtained from 4,5,6,7-tetrahydro-benzo[*b*]thiophen-4-one as a starting material (**1**). Various amino groups were introduced to obtain a large sampling of basicity and lipophilicity in the final structures. The second series, resembling the most to TCP itself, was issued from 4,5,6,7-tetrahydro-benzothiophen-7-one (**2**) with the aim to test the possible influence of the spatial position of the amino group. Finally, the third series was obtained from 5,6-dihydro-4*H*-thieno[2,3-*b*]thiopyran-4-one (**3**) as a starting material. Formally it results from the introduction of a second sulfur atom in the first series. According to MOPAC computations and to literature [25] this sulfur atom is able to readily oxidize to a stable dioxide which would lower the lipophilicity. This might help the clearance of such compounds.

The well-known ketone **1** was synthesized according to the method published by Cagniant et al. [26] (see *figure 3*). Ketone **2** was obtained starting from **1** by means of a Wolf–Kischner reduction followed by a regioselective oxidation of the resulting 4,5,6,7-tetrahydro-benzothiophene by means of Ce^{IV} in H_2O/AcOH (50:50) [27] (see *figure 3*). The oxidant addition must be strictly controlled because the mixture may rapidly turn black because of tars production. The best control was obtained when cooling the mixture at −15 °C. To get ketone **3**, 2-lithio-thiophen was first reacted with powdered sulfur to yield thiophen-2-thiol which was coupled to 4-bromopropylic acid to obtain 4-(2-thiophenyl)-4-thiobutyric acid. A final cyclization reaction in the presence of trifluoroacetic anhydride yielded ketone **3** [28] (see *figure 3*).

Amines belonging to the three series were obtained by a reductive amination of the suitable ketone (see *figure 2*). This reaction is usually done in a hydrochloric methanol solution [29, 30]. However, in our hands this way of doing acidification resulted in poor yields. The necessary acidification of the medium was best achieved by reacting amines in the form of their acetic salt. By this way we have obtained a good pH stabilization which facilitated the reductive amination. Finally, the best conditions of reaction were: 10 equiv. of acetate reacted with 1 equiv. of ketone in the presence of $NaBH_3CN$ in methanol at 80 °C. When using the commercial ammonium acetate the reaction was done at room temperature to avoid dimer formation. In our conditions the use of a dehydrating agent to facilitate the imine formation or pyridine to help the amine liberation never improved the yield. Finally a large excess of $NaBH_3CN$ was the single parameter able to improve really the yield; thus the reduction step seems to be the limiting factor in our conditions and not the iminium formation step. The compounds finally obtained were all checked by ^{1}H-NMR and/or ^{13}C-NMR spectroscopy (see table III and experimental part).

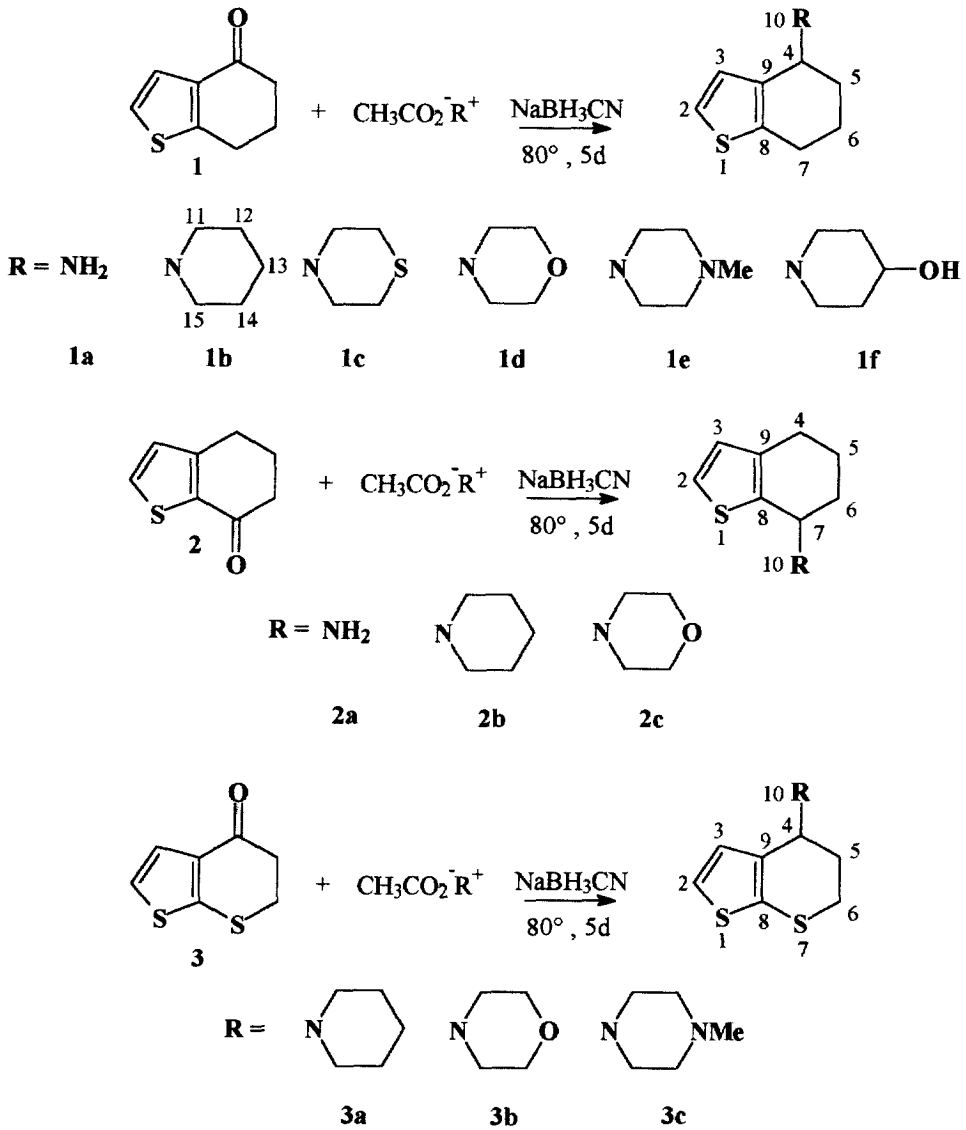

Figure 2.

3. Pharmacology

$O_2^{-\bullet}$ and $OH^{\bullet}$ radicals may be produced in vitro by means of various reagents or conditions: xanthine/xanthine oxidase (X/XO), hydrogen peroxide (H_2O_2), hypoxia, glucose stress for example [15, 31]. However these methods are not qualitatively, quantitatively, and kinetically equivalent. We have chosen the enzymatic reaction yielded by the pair X/XO because it allows a continuous liberation of $O_2^{-\bullet}$. Moreover this method appeared rapid enough to avoid cells reaction to stress which would decrease the glutamate reuptake inhibition. However, since X/XO alone is unable to yield $OH^{\bullet}$ radicals we have hypothesized that a Fe-catalysis according to the Haber–Weiss reaction could take place in the culture medium. Indeed it was likely that enough Fe ions

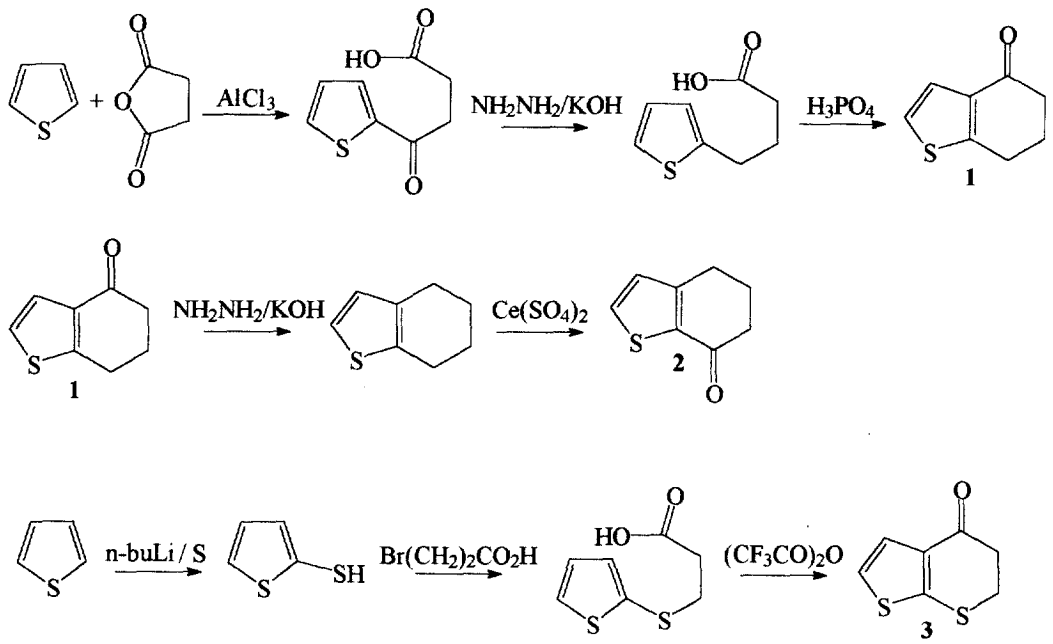

Figure 3.

originating from various endogenous and exogenous sources [13, 32] could be present and allow for the anticipated catalytic production of $OH^{\bullet}$ radicals.

4. Results and discussion

4.1. $OH^{\bullet}$ scavenging properties

The capacity of the newly obtained compounds to scavenge $OH^{\bullet}$ radicals was assessed by means of the modified method of Gutteridge [33]. This colorimetric assay allows for the computation of the $OH^{\bullet}$ scavenging rate constant (k_s) of a compound from the competitive inhibition of the 2-deoxyribose degradation induced by radicals. The medium contained EDTA to protect reagents against a possible iron chelation [36, 37]. Well-known $OH^{\bullet}$ scavengers like D-mannose, vitamin C, and salicylic acid were tested as reference compounds as well as the TCP model itself (see *table I*). TCP and new compounds were better competitors for $OH^{\bullet}$ scavenging than D-mannose and vitamin C. Compounds obtained from 4,5,6,7-tetrahydro-benzothiophen-4-one (**1**) and bearing an amino group constituted with a piperidine, morpholine or 1-methyl-piperazine ring (**1b**, **1d**, **1e**) appeared more efficient than salicylic acid although only **1b** and **1d** were statistically different from it (Student's *t*-test, see *table I*). Compounds issued from 4,5,6,7-tetrahydro-benzothiophen-7-one (**2**) displayed similar efficacy than salicylic acid. The last series issued from 5,6-dihydro-4*H*-thieno[2,3-*b*]thiopyran-4-one (**3**) also gave compounds with scavenging capacity in the same range as salicylic acid. Globally, in this chemical assay, all the new compounds were able to scavenge $OH^{\bullet}$ radicals with the same efficacy as salicylic acid, with the exception of **1b** and **1d**.

4.2. Affinity for the PCP receptor

Affinities of the compounds for rat brain membranes labeled with [^{3}H]TCP were determined by competition experiments [34]. As anticipated for rigid structures the affinities were negligible when compared to that of TCP (9.4 nM) since they varied between 14 μM and more than 100 μM (see *table I*). Thus the $OH^{\bullet}$ scavengers obtained are likely unable to interfere significantly with the glutamatergic transmission via the PCP receptor since the affinity of the best ligand **1e** (14 μM) for the PCP receptor is about 1500 times lower than that of TCP.

4.3. Protection of glutamate transporters in cultured astrocytes

We have first checked that the inhibition of [^{3}H]glutamate reuptake measured after 10 min exposure of

Table I. Rate constants [a] (k_s) of competition with 2-deoxyribose to scavenge OH• radicals, calculated log*P* and pK_a, and affinities [a] for the PCP receptor labeled with [^{3}H]TCP (IC_{50} and Hill numbers).

Compound	k_s (10^{-9} M^{-1} s^{-1}) (± SEM)	log*P* [b]	pK_a [b]	IC_{50} (μM) (± SEM)	n_H
D-mannose	0.94 ± 0.06			–	–
vitamin C	1.87 ± 0.17			–	–
TCP	2.75 ± 0.13			9.3×10^{-3}	1.00
salicylic acid	3.02 ± 0.18			–	–
1a	3.37 ± 0.25	1.81 ± 0.25	9.88 ± 0.2	29.5 ± 4.0	0.89
1b	3.61 ± 0.16 [c]	3.68 ± 0.28	9.07 ± 0.2	29.2 ± 7.8	0.79
1c	3.41 ± 0.25	3.01 ± 0.52	7.01 ± 0.2	> 100	–
1d	3.75 ± 0.33 [c]	2.13 ± 0.38	7.17 ± 0.2	> 100	–
1e	3.50 ± 0.23	1.86 ± 0.39	8.49 ± 0.7	14.0 ± 4.3	0.88
1f	3.27 ± 0.50	1.84 ± 0.36	8.31 ± 0.4	41.3 ± 2.7	0.81
2a	2.76 ± 0.06	1.81 ± 0.25	9.33 ± 0.2	> 100	–
2b	2.80 ± 0.26	3.68 ± 0.28	8.38 ± 0.2	19.8 ± 3.0	0.87
2c	3.33 ± 0.15	2.13 ± 0.38	6.48 ± 0.2	> 100	–
3a	3.45 ± 0.19	3.39 ± 0.80	8.77 ± 0.2	18.6 ± 2.9	1.18
3b	3.01 ± 0.14	1.84 ± 0.82	6.87 ± 0.2	> 100	–
3c	3.00 ± 0.19	1.57 ± 0.82	8.49 ± 0.7	54.7 ± 10.9	0.8

[a] Values resulted of at least 3 independent determinations.
[b] Calculated with the ACD/Log*P* and ACD/pK_a programs (ACD, Inc.) (95% confidence).
[c] Statistically more potent than salicylic acid, **2a**, and **2b** ($p < 0.05$).

cultured astrocytes to X/XO (500 μM/50 mU/mL) was due to a radical generation. According to Volterra et al. [15], the simultaneous addition of superoxide dismutase (SOD, 90 U/mL) and catalase (CAT, 3000 U/mL) completely prevented the [^{3}H]glutamate reuptake inhibition initiated by X/XO. However when SOD alone was practically unable to prevent X/XO inhibitory effect, CAT by itself protected the glutamate transporters against oxidation. Since SOD was ineffective by itself it is likely that $O_2^{-\bullet}$ did not participate directly to the glutamate transporters oxidation. Thus the [^{3}H]glutamate reuptake inhibition most probably resulted from the glutamate transporters oxidation by H_2O_2 and/or OH•. Secondly, we have studied the dose–response of [^{3}H]glutamate reuptake as a function of the radicals liberation by varying the X/XO concentrations (from 50 μM and 5 mU to 500 μM and 50 mU respectively). As expected the relationships between the radicals generation and the reuptake inhibition was found linear (not shown, $p < 0.0001$). The amount of X/XO (500 μM/50 mU) inducing about a 50% inhibition of [^{3}H]glutamate reuptake was chosen to challenge the potency of the scavengers (co-additioned with X/XO) to protect the transporters. Finally the effectiveness of compounds was expressed as the proportion of the reuptake protected from the basal 50% inhibition (% reuptake protected) at various concentrations (see *table II*, *figure 4*). Depending on their solubility and activity the compounds were tested in either of two ranges of concentrations: 0.3–1 mM or 1–400 μM. When plotting the % reuptake protected versus the concentration we have obtained three kind of dose–response curves typically represented in *figure 4*.

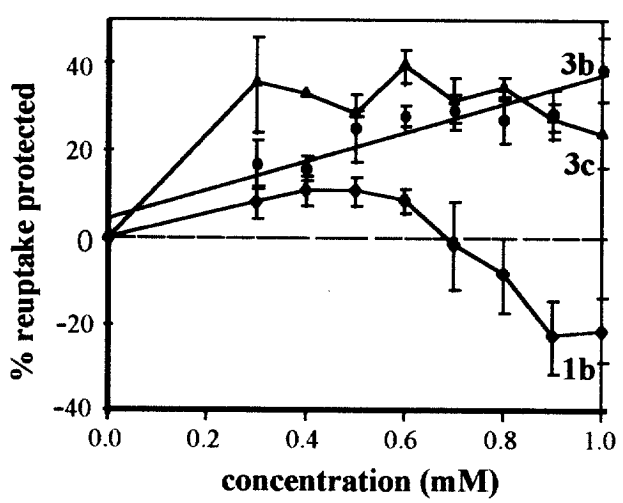

Figure 4. Typical dose–response curves representative of the protected (%) [^{3}H]glutamate reuptake in cultured astrocytes. The 50% reuptake inhibition level (0%) was achieved by 500 μM and 50 mU/mL of X/XO respectively.

*4.3.1. Linear dose–response curves (**1a, 1d, 1f, 2a, 2c, 3b**, see table II)*

The linear dose–response obtained with six compounds was in agreement with a radical scavenging process since the reuptake blockade was linearly related to radicals liberation (see above). Moreover it demonstrated that these compounds or their metabolites were little toxic for the reuptake process or astrocytes themselves in the dose range used (0.3–1 mM). As indicated above a plateau could not be reached because of compounds solubility. Three compounds were unable to protect the astroglial cells transporters against oxidation (**1a**, **1f**, **2a**). At the contrary three compounds protected 30–34% of the [^{3}H]glutamate reuptake at a 1 mM concentration (**1d**, **2c**, **3b**). Interestingly these compounds belonging to each of the three different series of molecules displayed a common feature: they all include a morpholine ring in their structure. With the exception of **1d,** statistically different from **2a**, the six compounds displayed similar scavenging properties in chemical assays (see *table I*) but large differences in biochemical assays (see *table II*). Interestingly, their calculated lipophilicities were similar since log*P*'s ranged from 1.81 ± 0.25 to 2.13 ± 0.38 (see *table I*). At the contrary, calculated pK_a's (see *table I*) revealed clear-cut differences. The pK_a's (6.48 ± 0.2 to 7.17 ± 0.2) of the potent compounds **1d**, **2c**, **3b** were significantly lower than the pK_a's (8.31 ± 04 to 9.88 ± 0.2) of the low active compounds **1a**, **1f**, **2a.** Therefore, in this case the protection against radicals in cultured astrocytes appeared dependent on basicity in addition to radical scavenging potency. In this respect, the beneficial role played by the morpholine ring might result from a good compromise between the basicity and the lipophilicity afforded by this amine to compounds **1d**, **2c**, **3b**.

*4.3.2. Bell shaped dose–response curves (**1b, 1c, 2b, 3a**, see table II)*

Four compounds gave such dose–response curves (**1b**, **1c**, **2b, 3a**). Compounds **1c**, **2b,** and **3a** displayed some efficacy (11–15% of [^{3}H]glutamate reuptake protected) at low concentrations when tested in the range 1–400 μM: the maximum of the reuptake protection was reached for concentrations about 50 μM (see *table II*). At the contrary **1b** was a poor protectant (tested in the 0.3 mM–1 mM range) although it appeared one of the most efficient compound in chemical assays (see *table I*). However the rapid decrease in protective action let suppose a 'toxic' action against the glutamate reuptake process or against astrocytes. The observed 'toxicity' might be due to molecules by themselves and/or their oxidation products. Alternatively these compounds might be more able than others to interact with astrocytes membranes. Indeed, they are formed with a piperidine (**1b**, **2b**, **3a**) or a thiomorpholine (**1c**) ring i.e. they are the most lipophilic compounds used in this work (3.01 ± 0.28 to 3.68 ± 0.28 log*P*, see *table I*). Thus they might bind to cell membranes by lipophilic interactions and be located close to the transporters. They could thus be protectants at low dosage but conversely they could modify the membrane structure to disrupt the reuptake function by a concentration dependent incorporation in the cellular membrane layers.

*4.3.3. Plateau dose–response curves (**1e**, **3c**, see table II)*

1e and **3c** gave linear dose–response curves at concentrations lower than 0.3 mM and a large plateau in the concentration range 0.3–1 mM. Globally they were rather good protectants since 24–32% of the [^{3}H]glutamate

Table II. Dose–response protection of glutamate transporters in cultured astrocytes against radicals generated by X/XO (500 mM/50 mU/mL).

Linear		Bell-shaped		Plateau	
Compounds	Slope [a] (% mM^{-1}) (r^2)	Compounds	Concentration (μM) and reuptake protected [b]	Compounds	Mean plateau (% ± SEM)
1d	34.1 (0.93) [c]	**2b**	50 (12% ± 4.2)	**3c**	31.8 ± 1.7
3b	33.1 (0.89) [c]	**1c**	50 (15% ± 4.5)	**1e**	24.5 ± 0.8
2c	31.9 (0.86) [c]	**3a**	50 (14% ± 2.6)	–	–
1f	9.1 (0.37)	**1b**	400–500 (11% ± 2.9)	–	–
1a	8.8 (0.42)	–	–	–	–
2a	8.3 (0.53)	–	–	–	–

[a] % of the [^{3}H]glutamate reuptake protected by a 1 mM concentration of each compound.
[b] Estimated at the maximum of the bell-shaped curves.
[c] $p < 0.001$.

Table III. ^{13}C-NMR chemical shifts of compounds (δ ppm from TMS in $CDCl_3$). Carbon numbering is according to *figure 2*.

	C Aromatic								C Aliphatic					
	C2	C3	C8	C9	C4	C5	C6	C7	C11	C12	C13	C14	C15	C16
1a	123.5	124.1	130.7	140.7	46.6	24.8	20.0	28.2						
1b	123.6	127.7	127.6	142.3	61.4	22.7	22.4	24.5	56.2	22.3	22.0	223	47.4	–
1c	124.0	127.7	127.0	142.7	62.8	24.5	22.2	24.8	53.0	48.6	–	48.6	53.0	–
1d	124.1	128.1	126.6	142.2	61.3	23.4	22.5	25.0	49.6	62.6	–	62.6	46.3	–
1e	124.7	126.0	127.1	143.8	61.7	21.9	21.6	24.3	42.7	49.7	–	49.4	42.4	46.2
1f	123.7	127.8	128.9	142.2	59.9	21.7	21.4	24.2	45.1	29.3	64.8	30.9	49.2	–
2a	125.4	128.5	132.1	139.7	29.5	20.0	26.4	46.6	–	–	–	–	–	–
2b	126.9	127.9	125.4	143.3	22.4	20.9	22.2	62.2	51.8	25.4	23.0	25.4	49.0	–
2c	127.3	128.1	124.6	143.6	25.3	25.0	20.5	62.2	63.7	48.5	–	48.3	63.3	–
3a	122.7	129.0	124.1	136.9	60.1	24.4	26.9	–	51.5	22.4	22.3	22.9	49.5	–
3b	122.1	130.1	124.0	136.9	59.1	22.2	23.9	–	50.3	63.3	–	63.0	47.8	–
3c [a]	122.3	129.5	124.7	136.0	58.9	22.8	24.9	–	42.2	49.9	–	49.9	41.0	46.7

[a] [d^6]DMSO.

reuptake was protected at the mean plateau (see *table II*). Interestingly **1e** and **3c** were the only compounds built with a 1-methyl-piperazine ring (see *figure 2*); thus, they are among the less lipophilic compounds prepared (1.57 ± 0.82 to 1.86 ± 0.38 log*P*, see *table I*). The large plateau observed at high concentrations was a likely pointing out of their low 'toxicity' for the reuptake process or astrocytes. However, the reason why these compounds were unable to protect more than about 30% of the reuptake whatever the dosage was remains unclear. The hypothesis that they protect only one of the astroglial transporters [35] is an attractive concept that requires additional study.

4.3.4. Radical generation by H_2O_2/Fe^{++}

In a last experiment we have used H_2O_2 in the presence of $FeSO_4$ for radical generation. The most efficient molecule **1d** (see *table II*) was unable to reverse the glutamate reuptake inhibition caused by 500 µM of H_2O_2 [15] alone since at a 1 mM concentration it increased unsignificantly (Student's *t*-test) the [^{3}H]glutamate reuptake from 68% to 75% (+ 7%) (see *figure 5*). At the contrary, when $FeSO_4$ was co-added with H_2O_2 to increase the $OH^\bullet$ concentration [38] in the culture medium, **1d** (1 mM) was able to increase significantly and dose-dependently the [^{3}H]glutamate reuptake from 61% to 84% (+ 23%) (see *figure 5*). The 16% reuptake difference between experiments was attributed to the scavenging of newly produced $OH^\bullet$ when Fe^{2+} was added. Inhibition of $OH^\bullet$ radicals is thus likely the way by which the newly obtained compounds were able to protect the glutamate transporters.

Finally, we have obtained new compounds able to interact with the $OH^\bullet$ radicals both in chemical medium (see *table I*) and in astrocyte cultures (see *table II*). Moreover they were devoid of significant affinity for the PCP receptor (see *table I*). Most of them displayed $OH^\bullet$ scavenging property in the same range as salicylic acid in chemical assays with the exception of **1b** and **1d** which were more efficient. Furthermore some of them were able to protect in vitro the glutamate reuptake in astrocytes by preventing the inactivation of glutamate transporters by radicals generated by X/XO. However, the protective potency in astrocyte cultures did not appear directly linked to the scavenging potency in chemical assays.

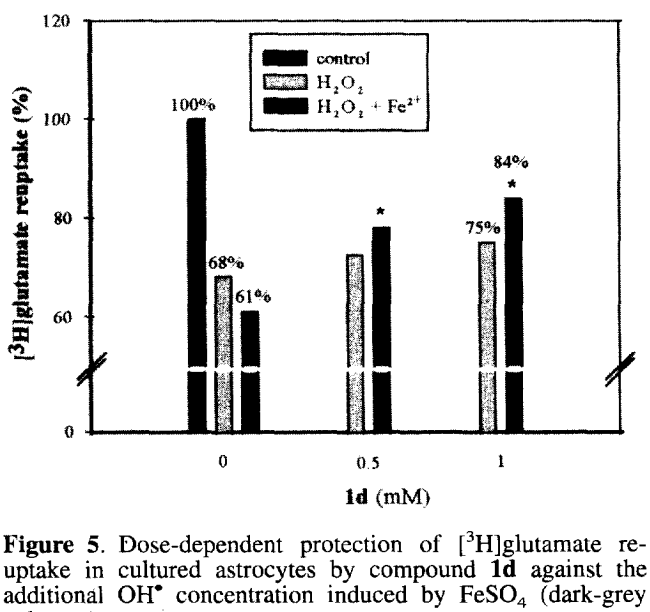

Figure 5. Dose-dependent protection of [^{3}H]glutamate reuptake in cultured astrocytes by compound **1d** against the additional $OH^\bullet$ concentration induced by $FeSO_4$ (dark-grey columns).

Interestingly their protective action against radicals was achieved according to various dose–response profiles which are in part at least dependent on the amine moiety. Since the molecules displayed very different profiles in cultured astrocytes and not in chemical assays, a role for physico-chemical differences revealed in the cultures may be suspected. Thus lipophilicity and basicity might hypothetically play an important role in the location with regard to membranes of these scavengers and modulate their ability to protect the transporters. For example, neither of hydrophilic and acidic molecules like Vitamin C [15] and salicylic acid (not shown) were able to counteract the effect of X/XO (500 μM/50 mU) in cultured astrocytes, though both were able to scavenge more or less $OH^{\bullet}$ radicals in chemical assays (see *table I*). Some of the new compounds might be interesting for in vivo neuroprotective assays. Therefore, the apparently most efficient compound **1d** is currently tested in our laboratory to assess its capacity to cross the blood brain barrier and to scavenge radicals inside the central nervous system.

5. Experimental protocols

5.1. Chemistry

Melting points (uncorrected) were determined with a Büchi–Tottoli apparatus. Yields were not optimized. Elemental analysis was performed at the CNRS Microanalytical Section in Montpellier on the hydrochloride salts and were within ±0.4% of theoretical values. ^{1}H- and ^{13}C-NMR spectra were obtained on a Brucker AC 200 spectrometer at 200.13 and 50.32 MHz respectively in 5-mm sample tubes in the FT mode. For some ^{13}C-signal assignments, a spin-echo sequence (Jmod) was used. Chemical shifts are reported in (δ) ppm downfield from TMS. Log*P* (octanol) and pK_a were calculated with the ACD/Log*P* and ACD/pK_a programs (ACD, Inc.).

5.1.1. $OH^{\bullet}$ scavenging assays

The modified method of Gutteridge described in [33] was used. Briefly, 2-deoxyribose (0.5 mM) and the scavenger (0.05–0.5 mM) were dissolved in a phosphate buffer (30 mM Na_2HPO_4, $7H_2O$, 40 mM NaCl, 0.1 mM EDTA) to reach a total volume of 500 μL. The reaction was initiated by addition of an aqueous solution of $FeSO_4$ to give a final concentration of 0.1 mM. After 15 min at 0 °C, 250 μL each of aqueous solutions of trichloroacetic acid (3%) and thiobarbituric acid (1.5%, dissolved at 100 °C) was added. Then the color developed. The absorbance was read at room temperature at 532 nm. The linear relationship $A_0/A = 1 + k_s/k_{DR}$ [(competitor)/(2-deoxyribose)] was used. A_0 and A are the absorbances in the absence or in the presence of the competitor respectively, k_{DR} and k_s are the 2-deoxyribose rate constant ($1.9\ M^{-1}\ s^{-1}$) and the competitor rate constant with $OH^{\bullet}$ respectively.

5.1.2. Synthesis of 4-oxo-4-thiophen-2-yl-butyric acid

Thiophene (2.85 mL, 0.036 mole) dissolved in 17 mL of CH_2Cl_2 was added dropwise under a nitrogen atmosphere to a solution of succinic anhydride (3.57 g, 0.036 mole) and $AlCl_3$ (10.46 g, 0.036 mole) in 35 mL of CH_2Cl_2. The mixture was stirred 5 h at room temperature, hydrolyzed by the successive addition at 0 °C of 25 mL of cold HCl (36%) and 20 mL of cold water. The mixture was extracted twice with CH_2Cl_2. The combined organic layers were extracted with NaOH solution washed with CH_2Cl_2. After acidification (HCl), the aqueous phase was extracted with CH_2Cl_2, the organic layer, dried over $MgSO_4$, was evaporated under reduced pressure. Crystallization in water gave light yellow spangles (3.97 g, 60%). ^{1}H-NMR: 7.77 (d, 1H, $H_{5'}$); 7.66 (d, 1H, $H_{3'}$); 7.15 (m, 1H, $H_{4'}$); 3.28 (t, 2H, H_3); 2.82 (t, 2H, H_2). ^{13}C-NMR: 192.3 (C_4); 180.0 (C_1); 144.5 ($C_{2'}$); 133.7($C_{3'}$); 132.0 ($C_{5'}$); 128.1 ($C_{4'}$); 33.6 (C_3); 27.9 (C_2).

5.1.3. Synthesis of 4-thiophen-2-yl-butyric acid

Hydrazine 98% (2.74 mL, 0.07 mole) and KOH (3.69 g, 0.066 mole) were added to a stirred solution of 4-oxo-4-thiophen-2-yl-butyric acid (3.6 g, 0.019 mole) in diethylene glycol (42 mL). The mixture was heated 7 h at 180 °C, then diluted at room temperature in 200 mL of water. The resulting aqueous phase, after extraction with ether, was acidified (HCl) before extraction with ether. The organic layer, dried over $MgSO_4$, was evaporated in vacuum to yield a pale yellow oil (2.74 g, 85%). ^{1}H-NMR: 7.15 (d, 1H, $H_{3'}$); 6.96 (t, 1H, $H_{4'}$); 6.84 (d, 1H, $H_{5'}$); 2.93 (t, 2H, H_2); 2.45 (t, 1H, H_4); 2.04 (q, 2H, H_3). ^{13}CNMR: 143.6 (C_1); 127.3 ($C_{3'}$); 124.8 ($C_{5'}$); 123.0 ($C_{4'}$); 33.3 (C_2); 28.5 (C_4); 26.1 (C_3).

5.1.4. Synthesis of 4,5,6,7-tetrahydro-benzothiophen-4-one 1

A solution of 4-thiophen-2-yl-butyric acid (2.74 g, 0.023 mole) in 3.9 mL of acetic anhydride containing 0.08 mL of orthophosphoric acid (85%) was stirred and heated at 120 °C for 2 h 30 min. The solution after cooling in an ice bath was hydrolyzed with 20 mL of water and the ketone **1** extracted with CH_2Cl_2. The organic layer washed with a NaOH solution, then with water until pH 7, was dried over $MgSO_4$ and concentrated in vacuum. Crystallization in petroleum ether yielded a white solid (2.23 g, 65%). ^{1}H-NMR: 7.24–7.21 (d, 1H, d, 1H, H_2-H_3) J_{23} = 0.34 Hz; 3.04 (t, 1H, H_5); 2.57 (m, 1H, H_7); 2.22 (m, 1H, H_6). ^{13}C-NMR: 193.3.3 (C_4); 155.8 (C_9); 137.0 (C_8); 124.8 (C_2); 122.4 (C_3); 38.2 (C_7); 25.5 (C_5); 24.2 (C_6).

5.1.5. Synthesis of 4,5,6,7-tetrahydro-benzothiophene

Hydrazine (98%) (2 mL, 0.028 mole) and KOH (2.86 g, 0.024 mole) were added to a well stirred solution of 4,5,6,7-tetrahydro-benzo[*b*]thiophen-4-one (**1**) (2.31 g, 0.007 mole) in diethylene glycol (40 mL). The mixture was heated at 180 °C for 8 h then diluted at room temperature with 200 mL of water. The aqueous phase was extracted with ether. The pooled organic layers were repeatedly washed with small amounts of water, dried over $MgSO_4$, and concentrated in vacuum to yield a transparent oil (2 g, 95%). ^{1}H-NMR: 7.03–6.74 (d, 1H, d, 1H, H_2–H_3) J_{23} = 0.29 Hz; 2.75–2.63 (t, 2H, t, 2H, H_4–H_7); 1.82 (m, 4H, H_5–H_6).

5.1.6. Synthesis of 4,5,6,7-tetrahydro-benzothiophen-7-one 2

Ce^{IV} sulfate (32 g, 0.06 mole) dissolved in 140 mL of AcOH/H_2O (50:50, v/v) was carefully added dropwise to a solution cooled at −15 °C of 4,5,6,7-tetrahydro-benzothiophene (2 g, 0.0144 mole) in 70 mL of AcOH/H_2O (50:50, v/v). During the addition the mixture must keep a pale yellow–orange color. After stirring at −15 °C during 2 h the mixture was stirred for additional

2 h at room temperature. The intense yellow mixture obtained was diluted with 200 mL of water and extracted with ether. The organic layers washed with a NaOH 5% solution, water, were dried over $MgSO_4$ and concentrated in vacuum. The oil obtained was purified by chromatography (SDS Chromagel 70–200 μm) in petroleum ether/ether (60:40) to yield 1.6 g of pure ketone (72%). ^{1}H-NMR: 7.62–6.98 (d, 1H, d, 1H, H_2–H_3) J_{23} = 0.63 Hz; 2.89 (t, 2H, H_6); 2.62 (t, 2H, H_4); 2.18 (q, 2H, H_5). ^{13}C-NMR:170.8 (C_7); 153.1 (C_8); 136.3 (C_9); 133.7 (C_2); 128.2 (C_3); 38.1 (C_6); 25.9 (C_4); 24.3 (C_5).

5.1.7. Synthesis of 3-(thiophen-2-yl-sulfanyl)propionic acid

n-Butyl-lithium 1.6 M in hexane (49 mL, 0.079 mole) was added dropwise to a stirred solution of thiophene (6.7 mL, 0.083 mole) in THF (40 mL) cooled at 0 °C. During the addition the temperature must be kept below 20 °C. After stirring for 1 h at 0–5 °C, powdered sulfur (2.51 g, 0.079 mole) was added in 20 min. After stirring during two additional hours, the mixture was hydrolyzed with 16 mL of water and purged with a nitrogen flow. The intense yellow mixture obtained was kept at 10 °C. In a second vessel, purged with a nitrogen flow, 3-bromopropanoic acid (10.94 g) was added to a solution of K_2CO_3 (4.8 g) in 16 mL of water and stirred at room temperature until it clarified. This solution was then slowly added to the previous one keeping the temperature below 5 °C. The resulting green aqueous phase was stirred for 24 h at room temperature. The aqueous phase was concentrated under a reduced pressure to eliminate THF, washed with toluene, acidified with of HCl 16 N (16 mL), and extracted 3 times with toluene (40 mL). The pooled organic phases were concentrated in vacuum and the remaining water azeotropically eliminated with additional toluene (20 mL) to yield a pale yellow oil (13.5 g, 86%). ^{1}H-NMR: 9.61 (s, 1H, H^+); 7.39 (d, 1H, $H_{5'}$); 7.18 (d, 1H, $H_{3'}$); 7.0 (q, 1H, H_4); 3.02 (t, 1H, H_3); 2.68 (t, 1H, H_2). ^{13}C-NMR: 177.8 (C_1); 134.8 ($C_{5'}$); 132.7 ($C_{2'}$); 130.1 ($C_{3'}$); 127.6 ($C_{4'}$); 34.2 (C_3); 33.1 (C_2).

5.1.8. Synthesis of 5,6-dihydro-4H-thieno[2,3-b]thiopyran-4-one ***3***

3-(Thiophen-2-yl-sulfanyl)propionic acid (13.5 g, 0.072 mole) was slowly added to trifluoroacetic anhydride (11 mL). The black mixture obtained was stirred at 36 °C for 3 h 30 min, hydrolyzed with water (50 mL) and with a NaOH solution until pH 9. The ketone was extracted with toluene, washed with water and concentrated in vacuum. The crude oily compound was rapidly purified by chromatography (SDS Chromagel 70–200 μm) in petroleum ether/ether (90:10) to give 10 g of a solid yielding white crystals of pure ketone **3** after crystallization in petroleum ether (8.5 g, 70%). ^{1}H-NMR: 7.46–7.02 (2d, 2H, H_2–H_3); 3.37 (t, 1H, H_6); 2.87 (t, 2H, H_4).

5.1.9. Amine preparation: general procedure

Acetic acid salts of 4-methyl-piperazine, piperidine, morpholine, and thiomorpholine: acetic acid (1 equiv.) dissolved in ether was added dropwise to a solution of amine (1 equiv.) in ether in a nitrogen atmosphere. The salt was stirred 1 h, the solvent evaporated, and the salt dried in vacuum at 40 °C for 1 night. 4-hydroxypiperidine was used as its hydrochloric salt formed by adding the amine solubilized in ether to a 3N HCl solution in ether and treated as above.

The amine salt (10 equiv.) prepared as above and $NaBH_3CN$ (0.7 equiv. or 1.5 equiv. when specified) were added to a solution of ketone **1**, **2** or **3** (1 equiv.) dissolved in MeOH (20 mL/1 equiv.). The mixture was stirred at 80 °C for 5 days (7 days at room temperature when ammonium acetate was used to obtain primary amines) and then hydrolyzed with cold water. The aqueous phase was concentrated under reduced pressure to eliminate MeOH, the pH adjusted to 9, and extracted with ether. The amine was then extracted in a HCl solution (pH = 1), washed with ether, made basic (NaOH solution), and extracted with ether. The pooled organic layers were dried over $MgSO_4$ and concentrated in vacuum to give the crude amine purified as specified below. The hydrochlorides of pure amines were obtained by adding them to anhydrous HCl solution in ether (3 N).

1a: The crude product was purified by column chromatography (Neutral Aluminiumoxide, activity I, Merck) in CH_2Cl_2/MeOH (99:1) (0.5 g, 88%). $M.p._{\cdot HCl}$ = 224.5–225.3 °C. Anal. $C_8H_{11}N$•HCl (C,H,N).

1b: The crude product was purified by column chromatography (Neutral Aluminiumoxide, activity I, Merck) in petroleum ether/ether (50:50) (0.4 g, 73% with 1.5 equiv. of $NaBH_3CN$). M.p. = 182–183 °C. Anal.$C_{13}H_{19}NS$•HCl (C,H,N,S).

1c: The crude product was purified by column chromatography (Neutral Aluminiumoxide, activity I, Merck) in petroleum ether/ether (60:40) (1.0 g, 63%). M.p. = 201.7–201.9 °C. Anal. $C_{12}H_{17}NS_2$•HCl (C,H,N,S).

1d: The crude product was purified by crystallization in ether (0.7 g, 99%, with 1.5 equiv. of $NaBH_3CN$). M.p. = 197–197.3 °C. Anal.$C_{12}H_{17}NOS$•HCl (C,H,N,O,S).

1e: The crude product was purified by column chromatography (Neutral Aluminiumoxide, activity I, Merck) in petroleum ether/ether (60:40) (0.85 g, 50%). M.p. = 176 °C. Anal. $C_{13}H_{20}N_2S$•2HCl (C,H,N,S).

1f: The crude product was purified by column chromatography (Neutral Aluminiumoxide, activity I, Merck) in petroleum ether/ether (80:20) (0.74 g, 47%). M.p. = 220.4–220.7 °C. Anal. $C_{13}H_{19}NOS$•HCl (C,H,N,O).

2a: The crude product was purified by column chromatography (Neutral Aluminiumoxide, activity I, Merck) in CH_2Cl_2/MeOH (99:1) (1.1 g, 42%). M.p. = 205–205.5 °C. Anal. $C_8H_{11}NS$•HCl (C,H,N,S).

2b: The crude product was purified by column chromatography (Neutral Aluminiumoxide, activity I, Merck) in petroleum ether/ether (50:50) (0.7 g, 60% with 1.5 equiv. of $NaBH_3CN$). M.p. = 171–171.7 °C. Anal. $C_{13}H_{19}NS$•HCl (C,H,N,S).

2c: The crude product was purified by column chromatography (Neutral Aluminiumoxide, activity I, Merck) in petroleum ether/ether (60:40) (0.88 g, 58% with 1.5 equiv. of $NaBH_3CN$). M.p. = 172.6–172.7 °C. Anal. $C_{12}H_{17}NOS$•HCl (C,H,N,O,S).

3a: The crude product was purified by column chromatography (Neutral Aluminiumoxide, activity I, Merck) in petroleum ether (0.95 g, 70% with 1.5 equiv. of $NaBH_3CN$). M.p. = 177.9–178.1 °C. Anal. $C_{12}H_{17}NS_2$•HCl (C,H,N,S).

3b: The crude product was purified by crystallization in ether (1.5 g, 65% with 1.5 equiv. $NaBH_3CN$). M.p. = 192.8–192.9 °C. Anal. $C_{11}H_{15}NOS_2$•HCl (C,H,N).

3c: The crude product was purified by column chromatography (Neutral Aluminiumoxide, activity I, Merck) in petroleum ether/ether (60:40) (1.77 g, 77% with 1.5 equiv. of $NaBH_3CN$). M.p. = 156.8–157.5 °C. Anal. $C_{12}H_{18}N_2S_2$•2HCl (C,H,N,S).

5.2. Pharmacology and biochemistry

5.2.1. Binding assays

[^{3}H]TCP (Amersham, 48 Ci/mmol, custom synthesis) binding to the PCP receptor was measured as previously described [34]. Briefly, the rat (Wistar) brain (minus the cerebellum) was homogenized with an Ultraturax (Ika Werke, maximum setting) in a 50 mM Tris/HCl, pH 7.7 buffer for 20 s at 4 °C. The homogenate was then centrifuged at 49 000 *g* for 20 min. The pellet was resuspended in the same buffer and the homogenization–centrifugation steps performed a second time. The final pellet was resuspended in 10 volumes of a 50 mM Tri/Hepes, pH 7.7 buffer and used without further purification. The homogenate (0.5–0.8 mg protein/mL) was incubated with [^{3}H]TCP (1 nM) in a 5 mM Tris/Hepes, pH 7.7 buffer (0.5 mL) in the absence (total binding) or in the presence of the competing drug for 60 min at 25 °C. The incubation was terminated by filtration over GF/B (Whatman) glass fibre presoaked in 0.05% polyethyleneimine (Aldrich) with an MR24 Brandel cell harvester. The filters were rinsed three times with 5 mL 50 mM NaCl, Tris HCl 10 mM, pH 7.7 buffer and the radioactivity retained was counted in 3.5 mL ACS (Amersham) with an Excel 1410 (LKB) liquid scintillation spectrophotometer. The non-specific binding was determined in parallel experiments in the presence of 100 μM unlabelled TCP.

5.2.2. Primary cultures of astrocytes

Primary astroglial cortical cell cultures were obtained from 2-days-old Sprague–Dawley rats (IFFA-Credo, France). The cerebral hemispheres were rapidly dissected in Hank's BSS (Gibco) supplemented with glucose (6 g/L), incubated in trypsine-EDTA (30 min at 35 °C), and the trypsine action stopped by 10% decomplemented fetal bovine serum. The cells were suspended and mechanically dissociated in the culture medium consisting of 87% Eagle's minimal essential medium (MEM, Gibco), 10% decomplemented fetal bovine serum and 0.6% glucose (without antibiotics), and plated on poly-D-lysine coated coverslips, in 24-well dishes, to a final mean density of 4×10^4 cells/cm^2 in 400 μL/well. The cells were maintained for 18 days at 35 °C in a humidified atmosphere (95% air, 5% CO_2), the medium being changed two times a week. The last change was without decomplemented fetal bovine serum because it apparently contained unidentified antiradical agents which interfered with experiments. The cells were vimentine and GFAP immuno reactive and had a flattened morphology. An optical control revealed that cultures contained about 98% of protoplasmic astrocytes.

5.2.3. [^{3}H]glutamate reuptake and transporter protection assays

Each well was first washed during 10 min with a buffer made with NaCl (124 mM), KCl (4.6 mM), $CaCl_2$ (1.2 mM), $MgCl_2$ (1.3 mM), KH_2PO_4 (0.416 mM), $NaHCO_3$ (26.75 mM), glucose (10 mM) and adjusted to pH 7.4 with diluted HCl. The wells were then incubated during 10 min at 27 °C in the buffer supplemented with xanthine alone (500 μM, Aldrich) or with xanthine and the radical scavenger. The radical generation was started by the addition of XO (50 mU/mL, thymol free, Aldrich) or H_2O_2/$FeSO_4$ (500 μM each) and the incubation continued for 10 min. The radical generation was stopped by 3 successive washes with CAT (3000 U/mL, Aldrich). The supernatant was changed for a 40 μM buffer solution of glutamate supplemented with [^{3}H]glutamate (56 Ci/mmol, isotopic dilution 1:25000, Amersham). The glutamate concentration was chosen near the K_m value for glutamate reuptake in our preparations. After 10 min incubation each well was rapidly washed 3 times with cold buffer then incubated few minutes in a water solution of NaOH (0.2 N). The cells were scraped and the radioactivity retained was counted in 3.5 mL ACS (Amersham) with an Excel 1410 (LKB) liquid scintillation spectrophotometer.

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