Available online at www.sciencedirect.com

SCIENCE DIRECT

European Journal of Pharmacology 512 (2005) 139–151

www.elsevier.com/locate/ejphar

Rapid and long-lasting tolerance to clomethiazole-induced hypothermia in the rat

Sandra A.G. Visser[a,*], Susanna Pozarek[a], Sofia Martinsson[b], Tomas Forsberg[b], Svante B. Ross[c], Johan Gabrielsson[a,1]

[a]PK/PD section, DMPK&BAC, Local Discovery Research Area CNS & Pain Control, AstraZeneca R&D Södertälje, SE-151 85 Södertälje, Sweden
[b]General Pharmacology, Local Discovery Research Area CNS & Pain Control, AstraZeneca R&D Södertälje, SE-151 85 Södertälje, Sweden
[c]Bioscience, Local Discovery Research Area CNS & Pain Control, AstraZeneca R&D Södertälje, SE-151 85 Södertälje, Sweden

Received 23 September 2004; received in revised form 4 February 2005; accepted 22 February 2005
Available online 28 March 2005

Abstract

Mechanism, onset and duration of tolerance development to clomethiazole-induced hypothermia were investigated in rats using telemetry. The hypothermic effect of clomethiazole was completely abolished for 10 days after an s.c. injection of 300 μmol/kg and the effect returned to ~50% in 32 days. The γ-aminobutyric acid$_A$ (GABA$_A$) receptor agonist muscimol induced hypothermia at 88 μmol/kg without any (cross-) tolerance. GABA$_A$ receptor antagonists, bicuculline (5.4 μmol/kg) and picrotoxin (3.3 μmol/kg), did not inhibit clomethiazole-induced hypothermia nor the tolerance. The noncompetitive *N*-methyl-D-aspartate (NMDA) receptor antagonist, dizocilpine, counteracted clomethiazole-induced hypothermia at 3 μmol/kg but not the tolerance. Tolerance to the 5-hydroxytryptamine$_{1A}$ (5-HT$_{1A}$) receptor agonist *R*-(+)-8-hydroxy-2-(di-*n*-propylamino)tetralin (R-8-OH-DPAT)-induced hypothermia was blocked by dizocilpine and clomethiazole but not vice versa. No pharmacokinetic interaction was observed. In conclusion, long-lasting tolerance to clomethiazole-induced hypothermia does not involve GABA$_A$ or 5-HT$_{1A}$ receptor functions. Glutamate via NMDA receptors may be involved in the hypothermic response but not in the tolerance.

Keywords: Clomethiazole; Body temperature; Tolerance; Pharmacodynamic interaction; GABA$_A$ (γ-Aminobutyric acid$_A$) receptor; 5-HT$_{1A}$ (5-Hydroxytryptamine$_{1A}$) receptor; NMDA (*N*-methyl-D-aspartate) receptor; Hypothermia

1. Introduction

Clomethiazole has sedative, hypnotic, anticonvulsive and neuroprotective properties and has been used clinically in elderly patients as a useful sedative/hypnotic for 40 years (Evans et al., 1986; Green, 1998). Several in vitro studies have shown that clomethiazole interacts with the γ-aminobutyric acid$_A$ (GABA$_A$) receptor–chloride channel complex (Leeb-Lundberg et al., 1981; Harrison and Simmonds, 1983; Ogren, 1986; Hedlund and Ogren, 1987; Simmonds and Turner, 1987; Cross et al., 1989; Moody and Skolnick, 1989; Vincens et al., 1989; Zhong and Simmonds, 1997; Green, 1998). In summary, these studies show that clomethiazole potentiates the effects of GABA and increases the chloride channel opening, thus appearing to modulate the GABA$_A$ receptor complex in a similar but not identical way to benzodiazepines and barbiturates (Cross et al., 1989; Zhong and Simmonds, 1997). In vivo studies have verified that clomethiazole enhances GABA$_A$ receptor functions because it inhibits seizures induced by antagonists of the GABA$_A$ receptor complex, e.g., bicuculline, picrotoxin and pentylenetetrazole (Ogren, 1986; Green and Murray, 1989).

Like other compounds that enhance GABA$_A$ receptor functions, clomethiazole induces hypothermia in rodents. For example, the GABA$_A$ receptor agonist muscimol induces hypothermia, which can be inhibited by bicuculline

* Corresponding author. Tel.: +46 8 553 24436; fax: +46 8 553 27491.
E-mail address: sandra.visser@astrazeneca.com (S.A.G. Visser).
[1] Current address: DMPK&BAC, AstraZeneca R&D Mölndal, SE-431 83 Mölndal, Sweden.

0014-2999/$ - see front matter © 2005 Elsevier B.V. All rights reserved.
doi:10.1016/j.ejphar.2005.02.036

and picrotoxin (Zarrindast and Oveissi, 1988). These results indicate that the $GABA_A$ receptor is involved in the muscimol-induced hypothermia. It is known that muscimol, like clomethiazole, possesses neuroprotective properties (Shuaib et al., 1993). However, contradictory reports exist as to whether the clomethiazole-induced hypothermia plays a role in its neuroprotective effects. Neuroprotection without hypothermia in the gerbil was observed during steady-state plasma concentration of 10 μM for 7 h (Cross et al., 1991). In contrast, Chaulk et al. (2003) reported neuroprotection and a delayed moderate hypothermia starting 6 h after the start of a 24-h infusion of clomethiazole with steady-state concentrations of 24 μM. This neuroprotective effect could not be detected when temperature confounds were eliminated. The question was therefore raised of whether the $GABA_A$ receptor is involved in the clomethiazole-induced hypothermia.

Clomethiazole induces a rapid onset and long-lasting tolerance to the hypothermic effect. Rapid tolerance and cross-tolerance to hypothermic and motor impairment responses have been reported for $GABA_A$ receptor modulators such as ethanol, chlordiazepoxide and pentobarbital (Khanna et al., 1991a,b, 1992). The tolerance development was blocked by the noncompetitive *N*-methyl-D-aspartate (NMDA) receptor antagonists dizocilpine ((+)MK-801) and ketamine (Khanna et al., 1991a, 1992).

Other compounds that induce hypothermia are 5-hydroxytryptamine$_{1A}$ (5-HT_{1A}) receptor agonists such as *R*-(+)-8-hydroxy-2-(di-*n*-propylamino)tetralin (R-8-OH-DPAT). However, rapid tolerance to 5-HT_{1A} receptor-mediated hypothermia, corticosterone secretion and cage-leaving response has been observed upon repeated dosing of R-8-OH-DPAT (Renyi et al., 1992; Ross et al., 1992). These effects could be antagonized with NMDA receptor antagonists. Furthermore, it was also shown that the $GABA_A$ receptor chloride channel is also involved in long-lasting tolerance to R-8-OH-DPAT (Kelder and Ross, 1993, 2002). All the GABAergic compounds examined significantly counteracted the R-8-OH-DPAT-induced tolerance to the hypothermic response: muscimol, diazepam, pentobarbitone and clomethiazole (Kelder and Ross, 2002). Combined treatment of the rats with the $GABA_A$ receptor antagonist bicuculline or the $GABA_A$ receptor–chloride channel blockers picrotoxin and diazepam, pentobarbitone sodium, or clomethiazole significantly reduced the attenuation of the R-8-OH-DPAT-induced tolerance (Kelder and Ross, 2002).

Although the NMDA receptor antagonist, dizocilpine, is involved in 5-HT_{1A} receptor-mediated hypothermia, it cannot produce hypothermia on its own but rather impairs thermoregulation of rats in hot environments by blocking NMDA receptors (Canini et al., 2001). Based on the similar effects of dizocilpine and clomethiazole on the attenuation of the tolerance to R-8-OH-DPAT, it was hypothesized in the present investigation that the NMDA receptor and the 5-HT_{1A} receptor vice versa might also be involved in the $GABA_A$ receptor-mediated hypothermia and the subsequent development of tolerance.

Accordingly, the present investigation was aimed at characterizing the onset and duration of tolerance to the hypothermic effect of clomethiazole. It also addresses the questions of whether $GABA_A$, 5-HT_{1A} and glutamate NMDA receptor functions are involved in the development of this tolerance.

2. Materials and methods

2.1. Animal and housing

Male Sprague–Dawley rats (B&K Universal AB, Sollentuna, Sweden) were used. Upon receipt from the animal suppliers, the rats were acclimatized over a period of at least 5 days prior to surgery. Prior to surgery, they were held up to five per cage in transparent cages, with wood shavings as bedding. After surgery, they were kept one per cage. Throughout the experiment, the rats had access to food and tap water ad libitum. The room temperature was between 18 °C and 22 °C, with a relative humidity of between 40% and 80%. Air was refreshed 15–20 times per hour via a centrally placed air intake and peripheral ventilators and the artificial light followed a 12-h cycle (lights on at 6 AM). Ethical permission was obtained from the Animal Ethics Committee Stockholm, Sweden.

2.2. Implantation of telemetric device and carotid artery catheter

The rats were placed in a chamber, where a mixture of O_2/N_2O (1:1) containing 5% isoflurane (Baxter Medical AB, Kista, Sweden) was administered until they were anesthetized. Thereafter, a nose mask was used to maintain anesthesia with a mixture of O_2/N_2O (1:3) containing 2–3% isoflurane during surgery. A 1-cm midline abdominal incision was made and a radiotelemetry transmitter (TA10TA-F20, Data Sciences, St. Paul, MN, USA) was placed in the peritoneal cavity. The individual transmitter serial number was connected to a rat ID number before implantation. The peritoneal cavity was closed with a normal suture and cleaned with saline. The transmitter surgery was performed at least 7 days before dose administration.

For the pharmacokinetic experiments, satellite groups of rats with a catheter in the carotid artery and a transmitter in the peritoneal cavity were used. Polythene tubing (I.D. 0.58 mm, O.D. 0.96 mm) was used as catheter. Prior to insertion, the tubing was tested for leakage, placed in 70% ethanol for disinfection and flushed with saline. The catheter was inserted into the artery and tunneled subcutaneously to the neck. The throat wound was closed with wound clamps. The remaining part of the catheter was exteriorized using a rubber cylinder (Dacron Mesh Button, Instech Laboratories

Inc., Pennsylvania, USA), which was sewed to the skin. The catheter was flushed with heparinized saline and closed with a stop. All the surgery was performed 2 to 4 days before the kinetic experiment.

2.3. Compounds and doses

Clomethiazole (clomethiazole edisilate, MW 513.48 g/mol) was obtained from Compound Management (AstraZeneca R&D Södertälje, Sweden). The concentration in all subcutaneous injection solutions was calculated on the basis of the clomethiazole base (MW 161.7 g/mol). *R*-(+)-8-hydroxy-2-(di-*n*-propylamino)tetralin (R-8-OH-DPAT: MW 328.3 g/mol), dizocilpine (MK-801, MW: 337.4 g/mol), picrotoxin (MW 602.59 g/mol), (+)-bicuculline (MW 367.36 g/mol) and muscimol (MW 114.1 g/mol) were bought from Sigma-Aldrich, Germany. Physiological saline (9 mg/mL, Braun Medical AB, Bromma, Sweden) was used as vehicle and all injection solutions were made in saline, except for (+)-bicuculline, which was dissolved in a few drops of glacial acetic acid and further diluted with 5% glucose in saline. The pH of the 300 μmol/kg clomethiazole solution was 2.1 and was not adjusted to physiological pH due to the low solubility of clomethiazole at higher pH values. All solutions were made on the day of the experiment.

Alzet® osmotic pumps (model 2001D, Scanbur BK AB, Sollentuna, Sweden) with an averaged pump rate of 7.4 μL/h were used for subcutaneous drug administration at a continuous rate. The pumps were filled with a solution of clomethiazole or R-8-OH-DPAT in saline. Filling weight was recorded in order to check appropriate filling. The pumps were preincubated in saline for a minimum of 2.5 h at 37 °C before being inserted subcutaneously into the neck of the rat. At the time of dosing, the animals were anesthetized with isoflurane for 5–10 min. A small incision was made in the neck, the skin was separated from the subcutaneous tissue, and the pump was inserted. The incision was closed with two wound clamps and the time of insertion of the pump was recorded. Exactly 24 h later, the pump was removed under light isoflurane anesthesia. In Table 1, an overview is given of the administered doses.

2.4. Pharmacological study

Body temperature was recorded from 4 days before drug administration until 4–6 days after the end of drug administration. The telemetry signals were measured every 2 min for a 10-s period and signaled to a receiver. The receiver was connected to the computer through a Dataquest PCI card (Data Sciences). Using the software package Dataquest ART 2.2 (Data Sciences), the temperature data were processed, analyzed and visualized.

The rats were randomly assigned to dosing groups. They were weighed prior to dosing and received a weight-adjusted dose. All pretreatment dosing occurred 20 min before treatment except for pretreatment with muscimol, bicuculline and picrotoxin, which were injected at the same time as the treatment. To ensure complete washout of the first treatment, a challenge dose was injected after 48 h. The experimental dosing design for the characterization of the hypothermia and tolerance development is given in Table 2. The experimental design (and results) is summarized for the pharmacological interaction and cross-tolerance experiments in Tables 3–5. The animals were disturbed as little as possible throughout the experiment by allowing entry to the experimental room only during drug administration.

Table 1
Compounds and doses used for s.c. injections and osmotic pump

Compound	Dose	Route	Dose volume
Clomethiazole	15 μmol/kg	s.c. Injection	1.5 mL/kg
Clomethiazole	150 μmol/kg	s.c. Injection	1.5 mL/kg
Clomethiazole	300 μmol/kg	s.c. Injection	1.5 mL/kg
Clomethiazole	600 μmol/kg	s.c. Injection	1.5 mL/kg
Clomethiazole	20 μmol/h/kg	24-h Osmotic pump	680 μmol/mL
Clomethiazole	40 μmol/h/kg	24-h Osmotic pump	1350 μmol/mL
R-8-OH-DPAT	0.3 μmol/kg	s.c. Injection	1.0 mL/kg
R-8-OH-DPAT	3 μmol/kg	s.c. Injection	1.0 mL/kg
R-8-OH-DPAT	0.3 μmol/h/kg	24-h Osmotic pump	40 μmol/mL
Dizocilpine	3 μmol/kg	s.c. Injection	0.8 mL/kg
Dizocilpine	0.18 μmol/h/kg	24-h Osmotic pump	24 μmol/mL
Muscimol	8.8 μmol/h/kg	s.c. Injection	1.5 mL/kg
Muscimol	88 μmol/h/kg	s.c. Injection	1.5 mL/kg
Bicuculline	5.4 μmol/kg	s.c. Injection	1.5 mL/kg
Picrotoxin	3.3 μmol/kg	s.c. Injection	1.5 mL/kg

The drug delivery rate with the osmotic pump was 7.4 μl/h.

2.5. Pharmacokinetic study

Temperature recording was started on the day before the experiment. On the morning of the experiment, the arterial catheter was flushed with heparinized saline and connected to an extension catheter. The rats were placed in a cage with the exteriorized rubber cylinder connected a swivel to allow them to move freely. They were allocated to dosing groups of 3 to 6 rats. Blood samples were taken at regular intervals up to 2 mL of blood being withdrawn for the determination of exposure to clomethiazole, R-8-OH-DPAT, or dizocilpine alone and in presence of each other. The samples were collected in heparinized tubes and after centrifugation for 10 min at 4500 *g*, 50 μL of plasma was transferred to glass tubes and stored at −20 °C until high performance liquid chromatography (HPLC) analysis. Temperature measurements were stopped on the day after sampling. No satellite pharmacokinetic groups are available for muscimol, picrotoxin and bicuculline, because the temperature experiments were carried out during another study.

2.6. Clomethiazole plasma analysis

The plasma samples were analyzed for clomethiazole using an HPLC-UV analysis method. To a 50-μL plasma

Table 2
Experimental design and averaged area under effect curve relative to vehicle (AUEC, mean ± S.E.M.) for characterization of the hypothermia and tolerance development to clomethiazole (CMZ)

Group	Day	Compound dose $t=0$	AUEC 0–12 h	Day	Challenge dose	AUEC 0–12h	N	Weight (g)
Clomethiazole-induced hypothermia								
A1	1	VEH	0 ± 1	–	–	–	6	297 ± 17
A2	1	CMZ 15	1 ± 1	–	–	–	6	312 ± 3
A3	1	CMZ 150	−8 ± 2	–	–	–	6	318 ± 2
A4	1	CMZ 300	−18 ± 4[a]	–	–	–	6	317 ± 3
A5	1	CMZ 600	−40 ± 3[a]	–	–	–	5	318 ± 8
Onset and duration of tolerance to CMZ								
B1	1	VEH	0 ± 1	3	CMZ 300	−20 ± 2[b]	6	297 ± 3
B2	1	CMZ pump 20	−8 ± 2	3	CMZ 300	0 ± 1[c,d]	6	312 ± 4
B3	1	CMZ pump 40	−13 ± 1[b]	3	CMZ 300	0 ± 1[c,d]	6	308 ± 5
B4	1	CMZ 300	−16 ± 2[b]	3	CMZ 300	−2 ± 1[c,d]	6	298 ± 5
B5	1	CMZ 300	−26 ± 4[b]	6	CMZ 300	−2 ± 1[c,d]	6	304 ± 5
B6	1	CMZ 300	−20 ± 3[b]	10	CMZ 300	−2 ± 1[c,d]	6	290 ± 9
B7	1	CMZ 300	−17 ± 2[b]	16	CMZ 300	−5 ± 1[c,d]	6	301 ± 4
B8	1	CMZ 300	−20 ± 2[b]	24	CMZ 300	−5 ± 1[c,d]	6	297 ± 3
B9	1	CMZ 300	−16 ± 2[b]	32	CMZ 300	−11 ± 2[d,e]	6	298 ± 5

Body weight at time of first dosing is shown. All doses are denoted in μmol/kg.
[a] Significantly different from groups A1, A2 and A3 ($p<0.01$).
[b] Significantly different from VEH (B1 on day 1, $p<0.01$).
[c] Significantly less hypothermia than on day 1 ($p<0.001$).
[d] Significantly less hypothermia than group B1, day 3 ($p<0.001$).
[e] Significantly lower than group B4, B5, B6, B7, B8 on day 3 ($p<0.05$).

sample, 50 μL of internal standard PLT-137 (5-(2-bromoethyl)-4-methylthiazole, 10 μM, AstraZeneca R&D Södertälje, Sweden), 200 μL of buffer, and 2 mL of 10% n-hexane in diethyl ether were added. The tubes were vortexed (DADE, Multi-tube vortexer) for 10 min and subsequently centrifuged for 10 min at 4500 *g*. All the tubes were kept at −20 °C for at least 30 min. After freezing, the organic phase was decanted to new tubes containing 150 μL of buffer. The organic phase was evaporated for 7 min at 40 °C under a nitrogen gas pressure of 10–15 psi (Zymark TurboVap, Zymark corp. CA, USA). The injection volume was 60 μL. The HPLC system consisted of a Gynkotek 480 pump (Kovalent AB, Frölunda, Sweden) with a Gilson 234 autosampler (Pretech, Sollentuna, Sweden). The UV detection (255 nm) was carried out with a TSP spectra100 UV detector (Spectra

Table 3
Experimental design and averaged area under effect curve relative to vehicle (AUEC, mean ± S.E.M.) of pharmacological interaction and cross-tolerance between clomethiazole (CMZ), R-R-8-OH-DPAT (DPAT), dizocilpine (MK-801), bicuculline (BIC), picrotoxin (PIC) and muscimol (MUS)

Group	Pretreatment $t=-20$ min	Treatment $t=0$	AUEC 0–12h	Challenge dose on $t=48$ h	AUEC 0–12 h	N	Weight (g)
Pharmacological interaction with clomethiazole							
C1	VEH	CMZ 300	−26 ± 4	CMZ 300	0 ± 1	5	228 ± 3
C2	MUS 8.8[a]	CMZ 300	−27 ± 4	CMZ 300	−2 ± 1[b]	6	285 ± 4
C3	MUS 88[a]	CMZ 300	−169 ± 2	CMZ 300	–	6	345 ± 3
C4		MUS 88	−10 ± 2	CMZ 300	−31 ± 7[c]	5	294 ± 3
C5	BIC 5.4[a]	CMZ 300	−29 ± 3[d]	CMZ 300	−3 ± 1[b]	5	347 ± 10
C6	PIC 3.3[a]	CMZ 300	−31 ± 4[d]	CMZ 300	−2 ± 1[b]	6	320 ± 10
C7	DPAT 3	CMZ 300	−19 ± 2[d]	CMZ 300	−2 ± 1[b]	6	231 ± 3
C8	DPAT pump	CMZ 300	−26 ± 2[d]	CMZ 300	1 ± 1[b]	5	265 ± 6
C9		DPAT 3	−3 ± 1	CMZ 300	−30 ± 4[c]	6	239 ± 3
C10	MK-801 3	CMZ 300	−10 ± 2[e]	CMZ 300	−1 ± 1[b]	5	238 ± 3
C11	MK-801 pump	CMZ 300	−11 ± 3	CMZ 300	−6 ± 4[b]	3	272 ± 8
C12		MK-801 3	−3 ± 2	CMZ 300	−21 ± 6[c]	6	230 ± 4

All doses are denoted in μmol/kg. Body weight at time of first dosing is shown.
[a] Pretreatment was injected together with treatment at $t=0$.
[b] Not significantly different from response on day 3 for group C1, thus no tolerance prevention.
[c] Significantly different from response on day 3 for group C1 ($p<0.001$).
[d] Not significantly different from response on day 1 for group C1, thus no change in clomethiazole-induced hypothermia.
[e] Significantly different from group C1 on day 1 ($p<0.05$).

Table 4
Experimental design and averaged area under effect curve relative to vehicle (AUEC, mean ± S.E.M.) of pharmacological interaction and cross-tolerance between muscimol (MUS) and clomethiazole (CMZ)

Group	Pretreatment $t=-20$ min,	Treatment $t=0$	AUEC 0–12 h	Challenge dose on $t=48$ h	AUEC 0–12 h	N	Weight (g)
Pharmacological interaction with MUS							
D1	VEH[a]	MUS 88	−13 ± 1	MUS 88	−14 ± 4	6	290 ± 7
D2	CMZ 15[a]	MUS 88	−16 ± 2	MUS 88	−11 ± 1	5	294 ± 5
D3		CMZ 300	−24 ± 2[b]	MUS 88	−12 ± 1	6	309 ± 6

All doses are denoted in μmol/kg. Body weight at time of first dosing is shown.
[a] Pretreatment was injected together with treatment at $t=0$.
[b] Significantly different from groups D1 ($p<0.001$) and D2 ($p<0.01$).

Physics, CA, USA). Separation was performed using a prodigy 3 μ ODS (3) 100 A column (50 × 4.6 mm, Genetec, Frölunda, Sweden). The mobile phase consisted of 28% (v/v) acetonitrile in phosphate buffer (pH 1.5) with 1 mM sodium laurylsulphate. Peak heights of clomethiazole and internal standard were recorded and analyzed using the PE Nelson 900 system (Perkin Elmer, CA, USA) or Empower (Waters, MA, USA). The flow rate was 1.7 mL/min. Linear calibration curves were obtained for clomethiazole in the range 1–300 μmol/L ($r>0.994$, $n=9$) and the limit of quantification was 0.5 μmol/L for a 50-μL sample. The intra-assay coefficients of variation and precision for 2, 20 and 200 μmol/L were 28% and 89%, 5% and 94%, and 6% and 99% ($n=9$), respectively. The corresponding inter-assay figures were 15% and 97%, 12% and 94%, 7% and 95% ($n=18$), respectively. Recovery of extraction was 76% for clomethiazole.

2.7. R-8-OH-DPAT and dizocilpine plasma analysis

The plasma samples were analyzed for dizocilpine and R-8-OH-DPAT using reversed-phase liquid chromatography and electrospray tandem mass spectrometry. They were diluted to 200 μL with MilliQ water, of which 100 μL was used for extraction. Fifty microliter (10 nM ropivacaine) of internal standard and 50 μL of 0.1 M sodium hydroxide (NaOH) were added manually. The organic phase, 700 μL of (70/30%) heptane/ethyl acetate, was added using a 96-channel pipetting robot (Apricot Personal Pipettor, Perkin Elmer, Boston, USA). The samples were rotated for 10 min and centrifuged for 10 min at 5000 *g* (Rottixa 50RS, Hettich, Tuttlingen, Germany). The organic phase was transferred to a new plate using an 8-channel pipetting robot (Packard MultiProbe, Perkin Elmer, Boston USA) and evaporated to dryness under a stream of nitrogen (Micro-DS96, Porvair Sciences Ltd., Shepperton, UK) at 40 °C. The residue was dissolved in 30 μL of 0.01 M ammonium formate buffer pH 4.4. The plate was vortexed (Multi-tube Vortexer, Dade, Miami, Florida, USA) for 2 min and centrifuged for 3 min at 5000 *g*. The injection volume was 20 μL. The HPLC system consisted of two HPLC pumps from Shimadzu (Kyoto, Japan) with an autosampler from CTC Analytics AG (Zwingen, Switzerland). The flow rate was 0.23 mL/min. Separations were performed on a Zorbax Extend-C18 column (2.1 × 50 mm; 3.5 μm 80 Å) (ChromTech AB, Sweden). A guard column, hypurity Cyano (Javelin Guards and Filters, ChromTech AB, Sweden) was used. The mobile phase consisted of 0.01 M

Table 5
Experimental design and averaged temperature at $t=0.5$ h after injection (mean ± S.E.M.) of pharmacological interaction and cross-tolerance between R-R-8-OH-DPAT (DPAT) and clomethiazole (CMZ) and dizocilpine (MK-801)

Group	Pretreatment $t=-20$ min	Treatment $t=0$	Temperature $t=0.5$ h after injection	Challenge dose on $t=48$ h	Temperature $t=0.5$ h after injection	N	Weight (g)
Pharmacological interaction with DPAT							
E1	VEH	DPAT 3	34.6 ± 0.2	DPAT 0.3	36.4 ± 0.1[a,b]	6	248 ± 6
E2	VEH	DPAT 3	34.7 ± 0.3	DPAT 3	35.1 ± 0.3	4	241 ± 6
E3	CMZ	DPAT 3	33.7 ± 0.2[c]	DPAT 0.3	35.7 ± 0.2[b]	6	239 ± 6
E4	CMZ	DPAT 3	33.3 ± 0.3[c]	DPAT 3	35.2 ± 0.3[b]	4	244 ± 3
E5		CMZ 300	35.4 ± 0.2	DPAT 0.3	34.8 ± 0.3	4	248 ± 3
E6		CMZ 300	34.9 ± 0.2	DPAT 3	36.1 ± 0.4[d]	4	250 ± 4
E7	MK-801	DPAT 3	35.7 ± 0.4	DPAT 0.3	35.6 ± 0.2	6	265 ± 7
E8		MK-801 3	37.9 ± 0.1[e]	DPAT 0.3	34.7 ± 0.2[b]	2	249 ± 2
E9		MK-801 3	37.8 ± 0.3[e]	DPAT 3	35.6 ± 0.3[b]	6	239 ± 3

All doses are denoted in μmol/kg. Body weight at time of first dosing is shown.
[a] Significantly different from groups E2, E4, E5 ($p<0.01$) and E8, E9 ($p<0.05$) on day 3.
[b] Significantly different from treatment on day 1 ($p<0.002$) except group 8 ($p<0.05$).
[c] Significantly different from response on day 1 for group E7 ($p>0.001$).
[d] Significantly different from group E5 on day 3 ($p<0.05$).
[e] Significantly different from response on day 1 for groups E1, E2, E3, E4, E5, E6 ($p<0.001$) and E7 ($p>0.01$).

ammonium formate pH 4.4 in water/0.01 M ammonium formate pH 4.4 in acetonitrile (50/50). Dizocilpine and DPAT were detected by the mass spectrometer Quattro Micro from Waters (Manchester, UK) and chromatograms were processed using MassLynx 3.5 software from Waters (Manchester, UK). The limit of quantification was 3 nM for a 50-μL sample. Linear calibration curves were obtained for DPAT and dizocilpine in the range 3–300 nmol/L ($r>0.99$, $n=6$ and $r>0.99$, $n=4$). The inter-assay coefficients of variation and precision for 10, 50 and 250 μmol/L dizocilpine were 12% and 102%, 7% and 104%, and 6% and 95% ($n=4$), respectively. The corresponding inter-assay figures were 10% and 98%, 8% and 98%, 7% and 98% ($n=6$), respectively.

2.8. Statistical analysis

The area under the effect curve relative to vehicle was calculated by summation of individual temperatures every hour between 0 and 12 h (sum of 13 temperatures). In each group, all the individual areas were averaged (mean ± S.D.) and subtracted from the averaged area under the effect curves after vehicle injection (488 ± 1 °C between 0 and 12 h). The areas under the effect curves relative to vehicle were summarized in the tables for comparison between clomethiazole- and muscimol-induced hypothermia. Due to the short duration of R-8-OH-DPAT, it was decided to average the temperature 0.5 h after injection in order to compare R-8-OH-DPAT-induced hypothermia in each group. At this time-point, the minimum temperature was reached in all animals.

Statistical analysis was performed using one-way analysis of variance (ANOVA) and a Tukey–Kramer multiple comparison test. In case of nonhomogeneity, as determined by Bartlett's test, the nonparametric Kruskall–Wallis test was used. The repeated measurements on days 1 and 3 were tested using a paired students t-test. $P<0.05$ was considered significant. Statistical tests were performed using S-Plus (Insightful, Seattle, USA).

3. Results

3.1. Clomethiazole-induced hypothermia

Subcutaneous bolus injections of 15, 150, 300 and 600 μmol/kg clomethiazole induced an exposure-related decrease in the body temperature that lasted between 6 and 18 h (Fig. 1A). The corresponding averaged plasma concentrations are also shown in Fig. 1B. The calculated area under the effect curve relative to vehicle treatment is listed in Table 2. The area under the effect curve decreased dose-dependently and the areas for 300 and 600 μmol/kg (A4, A5) were significantly lower than vehicle or low-dose treatment (A1, A2, A3).

3.2. Onset and duration of tolerance to clomethiazole-induced hypothermia

The hypothermic effects following a 24-h continuous infusion of 0, 20 or 40 μmol/h/kg clomethiazole between $t=0$ and 24 h, followed by an s.c. bolus injection of 300 μmol/kg (exactly 24 h after removal of the osmotic pump), are depicted in Fig. 2, together with the plasma exposure to clomethiazole. There was a concentration-dependent decrease in temperature during the first 6 h of continuous clomethiazole infusion. However, despite constant exposure to clomethiazole, the temperature returned to the baseline value before the infusion was completed. The steady-state plasma concentrations of clomethiazole were 8 and 16 μM for the 20 and 40 μmol/h/kg infusion rates, respectively. A second injection of clomethiazole 24 h after removal of the pump did not induce hypothermia. Thus, the rapid and complete tolerance developed during continuous infusion of clomethiazole lasted at least 48 h. The control animals (triangles) that received a saline infusion between 0 and 24 h responded with a normal hypothermic response to a 300 μmol/kg injection of clomethiazole bolus at 48 h.

Fig. 3A shows the mean temperature profiles after a 300 μmol/kg injection of clomethiazole on day 1, followed by an

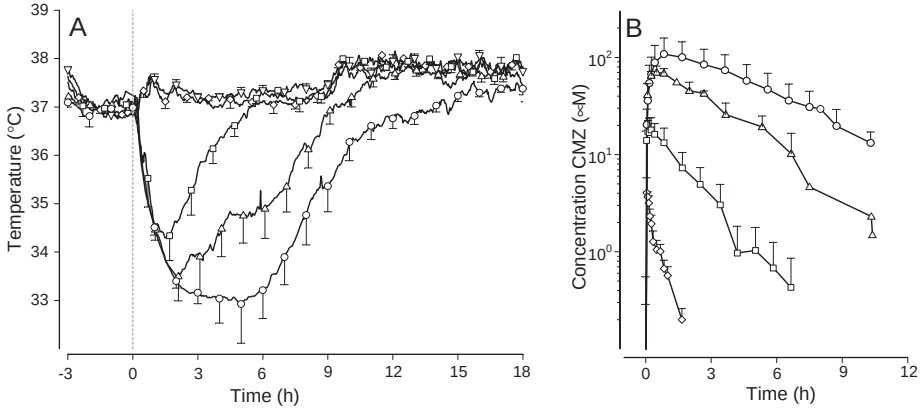

Fig. 1. Clomethiazole-induced hypothermia. (A) Averaged hypothermic response (mean ± S.E.M., groups A1–A5) and (B) averaged plasma concentrations (mean ± S.E.M., groups F1–F4) for clomethiazole. Subcutaneous doses of 0 (∇), 15 (◇), 150 (□), 300 (△), and 600 (○) μmol/kg clomethiazole were injected at time = 0 (10:00 AM).

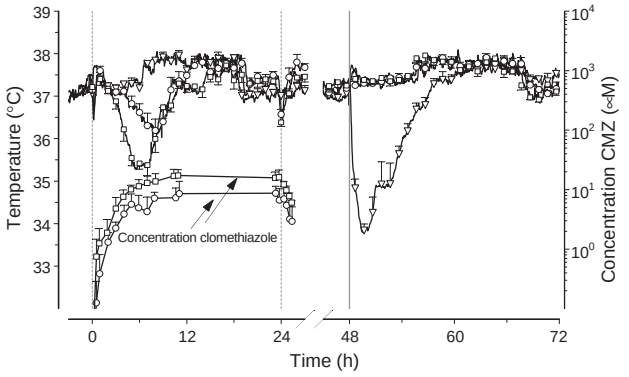

Fig. 2. Tolerance development to clomethiazole. Averaged effects (mean ± S.E.M., groups B1–B3) on body temperature and plasma concentrations (mean ± S.E.M., groups F5–F6, scaled to right *y*-axis) during 24 h of continuous s.c. infusion of 20 (○) and 40 μmol/h/kg clomethiazole (□) and 24 h of continuous infusion of saline (∇). Dotted vertical lines indicate implantation and removal of the osmotic pumps. At $t = 48$ h (vertical solid line) a 300 μmol/kg s.c. bolus injection of clomethiazole was given to all groups.

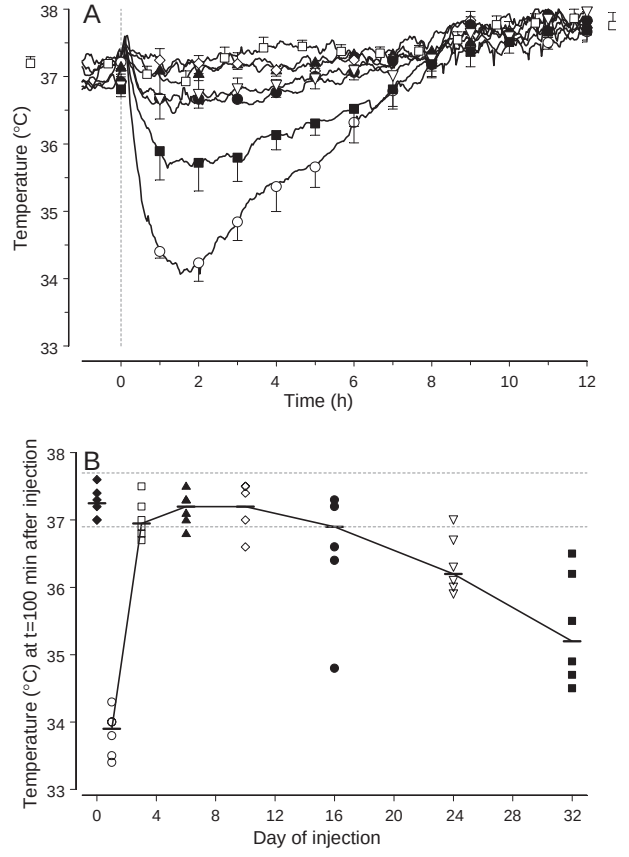

Fig. 3. Duration of tolerance to clomethiazole. (A) The effects of a 300 μmol/kg clomethiazole challenge injection on various days after an initial injection on day 1 (○), day 3 (□), day 6 (▲), day 10 (◇), day 16 (●), day 24 (∇) and day 32 (■) (groups B4–B9). Injection occurred at $t = 0$ (10:00 AM). (B) Individual temperatures measured 1.2 h after injection for the same groups as in the upper panel. On day 0, the temperature responses are depicted to an injection of saline (♦). The responses on day 1 and day 32 at 1.2 h after administration were significantly ($p < 0.05$) different from saline treatment. The line represents the median value of six animals and the dotted lines represent the variation within the saline injection group.

additional injection on day 3, 6, 10, 16, 24 or 32. The corresponding areas under the effect curves are listed in Table 2 (groups B4–B9). No hypothermic response was observed on days 3, 6 and 10 and the response to clomethiazole on day 32 was significantly larger than the response on day 3. All individual temperature decreases 100 min after injection are depicted in Fig. 3B. All animals responded on day 1 to a bolus injection of clomethiazole with a temperature decrease to 33.8 ± 0.3 °C. No effect was observed on days 3, 6 and 10, compared to control treatment (37.3 ± 0.2 °C). On days 16, 24 and 32 the decreases were to 36.6 ± 1 °C, 36.7 ± 0.4 °C and 35.4 ± 0.8 °C, respectively. Calculated on the basis of the maximum decrease at 1.5 h after injection or the area under the effect curve, the hypothermic response returned to 55% or 50% of the initial value in 32 days, respectively.

3.3. Influence of $GABA_A$ receptor agonist muscimol and antagonists bicuculline and picrotoxin on clomethiazole hypothermia and tolerance

Neither 5.4 μmol/kg bicuculline, nor 3.3 μmol/kg picrotoxin, nor 8.8 μmol/kg muscimol was able to alter the hypothermic response to clomethiazole compared to vehicle when given s.c. simultaneously at $t = 0$, as shown in Fig. 4. Challenging the same animals with clomethiazole administered 48 h later, no hypothermia was observed for these groups, irrespective of the different pretreatment with $GABA_A$ receptor agonist or antagonists, indicating a persistent tolerance to clomethiazole. A single injection of 88 μmol/kg muscimol induced hypothermia lasting for 8 h, with a minimum temperature of 35.5 °C. However, repeated injection of 88 μmol/kg muscimol did not show

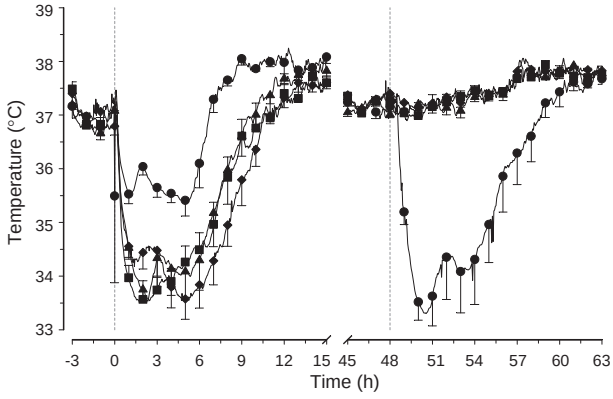

Fig. 4. Influence of GABAA receptor agonist muscimol and antagonists bicuculline and picrotoxin on clomethiazole hypothermia and tolerance. Averaged (mean ± S.E.M., $n = 5$–6, groups C2, C4, C5, C6) temperature responses upon s.c. injection of 300 μmol/kg clomethiazole with co-treatment of 5.4 μmol/kg bicuculline (■), 3.3 μmol/kg picrotoxin (♦), or 8.8 μmol/kg muscimol (▲) at $t = 0$ min. Circles (●) represent a single injection of 88 μmol/kg muscimol at $t = 0$. All groups received a challenge dose of 300 μmol/kg clomethiazole at $t = 48$ h. The dotted reference lines indicate time of injection of pretreatment, treatment and challenge dose, respectively.

any development of tolerance to its hypothermic effect, as observed for clomethiazole. A dose of 88 μmol/kg muscimol together with 300 μmol/kg clomethiazole resulted in a continuously decreasing temperature, eventually resulting in death (data not shown). In addition, no cross-tolerance was observed after single injection of muscimol at time zero and clomethiazole after 48 h and vice versa. Estimates of the area under the effect curve are depicted in Table 3. Subcutaneous dosing of muscimol, bicuculline and picrotoxin did not alter the clomethiazole-induced hypothermic response or the development of tolerance.

3.4. Influence of the 5-HT_{1A} agonist R-8-OH-DPAT on clomethiazole hypothermia and tolerance

The hypothermic response to injection of 300 μmol/kg clomethiazole was not altered by pretreatment of 3 μmol/kg R-8-OH-DPAT, as shown in Fig. 5. Challenging the same animals with clomethiazole 48 h later, no hypothermia was observed, indicating a persistent tolerance to clomethiazole. A single injection of 3 μmol/kg R-8-OH-DPAT gave a temperature decrease to 34 °C and lasted for 3–4 h. No cross-tolerance was observed after a single injection of R-8-OH-DPAT at time zero and clomethiazole after 48 h and vice versa. The average temperature profiles for groups C1, C7 and C9 are shown in Fig. 5. For reasons of clarity, the group that received a continuous s.c. infusion of R-8-OH-DPAT and a s.c. injection of clomethiazole (group C8) is not shown in the graph. Areas under the effect curve are depicted in Table 3. R-8-OH-DPAT has no influence on the hypothermic profile of clomethiazole and did not prevent the tolerance.

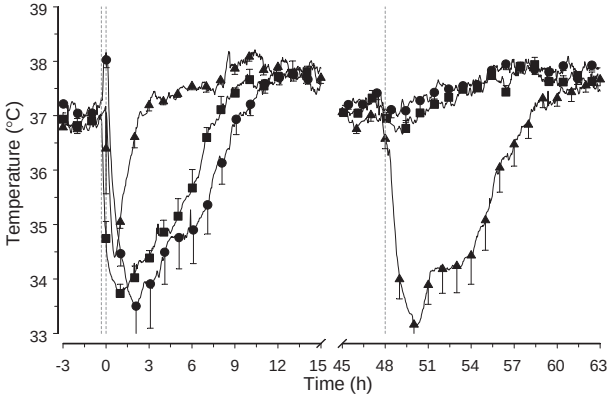

Fig. 5. Influence of the 5-HT1A agonist R-8-OH-DPAT on clomethiazole hypothermia and tolerance. Averaged (mean ± S.E.M., n = 5–6, groups C1, C7, C9) temperature responses upon s.c. injection of 300 μmol/kg clomethiazole at t = 0 with pretreatment of vehicle (●), 3 μmol/kg R-8-OH-DPAT (■) at t = −20 min. Triangles (▲) represent a single injection of 3 μmol/kg R-8-OH-DPAT at t = 0. A challenge dose of 300 μmol/kg clomethiazole was given to all groups at t = 48 h. The dotted reference lines indicate times of injection of pretreatment, treatment and challenge dose, respectively.

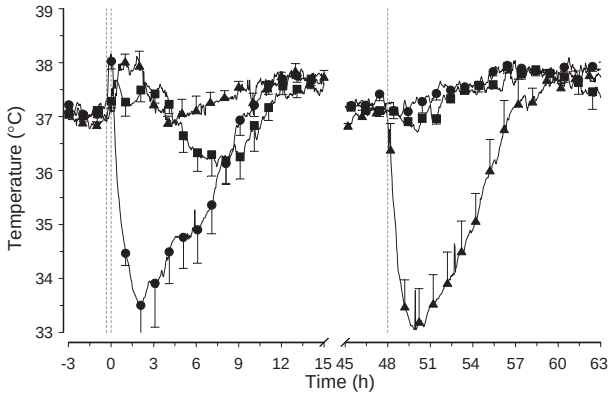

Fig. 6. Influence of NMDA receptor antagonist dizocilpine on clomethiazole hypothermia and tolerance. Averaged (mean ± S.E.M., n = 5–6, C1, C10, C12) temperature responses upon s.c. injection of 300 μmol/kg clomethiazole at t = 0 with pretreatment of vehicle (●), 3 μmol/kg dizocilpine (■) at t = −20 min. Triangles (▲) represent a single injection of 3 μmol/kg dizocilpine at t = 0. A challenge dose of 300 μmol/kg clomethiazole was given to all groups at t = 48 h. The dotted reference lines indicate times of injection of pretreatment, treatment and challenge dose, respectively.

3.5. Influence of the NMDA receptor antagonist dizocilpine on clomethiazole hypothermia and tolerance

Pretreatment with an s.c. injection of dizocilpine reduced the hypothermic response to an s.c. bolus injection of 300 μmol/kg of clomethiazole. However, when clomethiazole was given with a constant rate of dizocilpine, the temperature response varied rapidly between 36 and 35 °C during 12 h and was not completely blocked by dizocilpine (group C11, data not shown). Challenging the same groups of animals with clomethiazole after 48 h, no hypothermia was observed, indicating that dizocilpine did not prevent tolerance development to clomethiazole. A single s.c. injection of dizocilpine itself did not alter but rather slightly increased the body temperature in comparison to vehicle injection. No cross-tolerance was observed after a single injection of dizocilpine at time zero and clomethiazole after 48 h and vice versa. The average temperature profiles are shown in Fig. 6. Estimates for areas under the effect curve are depicted in Table 3. Although dizocilpine was able to block the hypothermic response to clomethiazole, it was unable to prevent the development of tolerance to clomethiazole.

3.6. Comparison of clomethiazole-, muscimol- and R-8-OH-DPAT-induced hypothermia and tolerance development

In contrast to the complete tolerance to the clomethiazole-induced hypothermia, the $GABA_A$ receptor agonist muscimol did not induce any tolerance to itself. Two s.c. injections 48 h apart induced similar hypothermic profiles, with a maximum decrease to 35 °C. The areas are depicted in Table 4.

R-8-OH-DPAT induced 100% tolerance during a continuous s.c. infusion of 0.3 μmol/kg/h by means of an

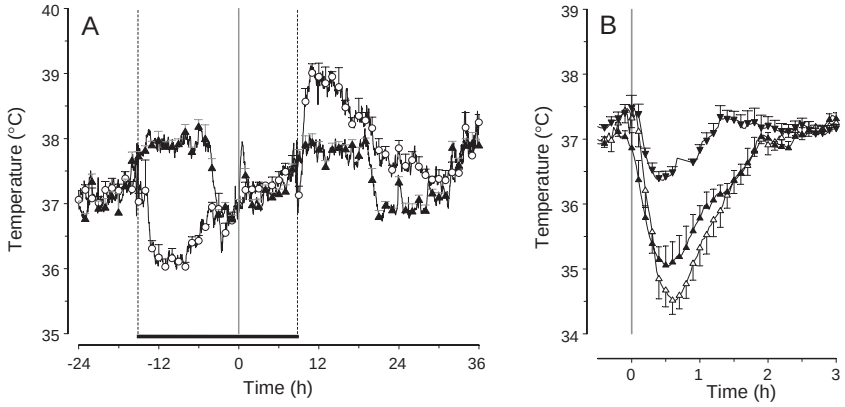

Fig. 7. Tolerance development to R-8-OH-DPAT during acute and continuous administration. (A) An osmotic pump was implanted between −15 and 9 h (dotted vertical lines, 6 PM–6 PM). At $t=0$ a saline injection was given (10 AM, solid vertical line). Vehicle-controlled profile (▲) and the temperature profile during R-8-OH-DPAT administration (○) are depicted. Complete tolerance to R-8-OH-DPAT occurred during 24-h administration, followed by a rebound increase in temperature lasting 24 h. (B) The average temperature response (mean ± S.E.M., $n=5$–6, E1, E2) to a first injection of 3 μmol/kg (△) and second injection of 3 μmol/kg (▲) or 0.3 μmol/kg (▼) R-8-OH-DPAT with a 48-h interval. The second injection of 3 μmol/kg R-8-OH-DPAT resulted in an 18% smaller hypothermic response than on the first day.

osmotic pump (see Fig. 7A). The pump was implanted at 6 PM and removed 24 h later ($t=0$ corresponds to 10 AM when vehicle injection was given). Compared to vehicle control, there was a substantial decrease in temperature, which gradually returned to baseline during drug administration. The exposure to R-8-OH-DPAT was measured between 10 AM and 6 PM and was found to be constant at 105 ± 12 nM. After removal of the pump, a pronounced rebound increase of 1 °C in body temperature was observed. The rebound period lasted for almost 24 h.

During the 24-h administration full tolerance development to R-8-OH-DPAT was observed; however, a challenge injection of 3 μmol/kg R-8-OH-DPAT 48 h after a first injection of 3 μmol/kg R-8-OH-DPAT showed only partial tolerance (group E2, Fig 7B). This reduction was more pronounced when the second injection of R-8-OH-DPAT was 0.3 μmol/kg (group E1, Fig 7B). For the first and second injections of 3 μmol/kg R-8-OH-DPAT, the minimum temperatures 30 min after injection were 34.7 ± 0.3 °C and 35.1 ± 0.3 °C, respectively (mean ± S.D., Table 5). The minimum temperature 30 min after injection of 0.3 μmol/kg R-8-OH-DPAT, injected 48 h after 3 μmol/kg R-8-OH-DPAT, was 36.4 ± 0.1 °C. The reduction in temperature decrease due to repeated dosing of 3 μmol/kg R-8-OH-DPAT was 18%, when taking 37 °C as baseline value.

3.7. Clomethiazole and dizocilpine can both attenuate tolerance to R-8-OH-DPAT

The temperature response following a s.c. injection of 0.3 μmol/kg R-8-OH-DPAT 48 h after administration of 300 μmol/kg clomethiazole together with 3 μmol/kg R-8-OH-DPAT (E3) and 300 μmol/kg clomethiazole (E5) was similar, whereas the response was smaller for group E1, which received 3 μmol/kg R-8-OH-DPAT 48 h earlier (see Fig. 8A). Thus, pretreatment with 300 μmol/kg clomethia-

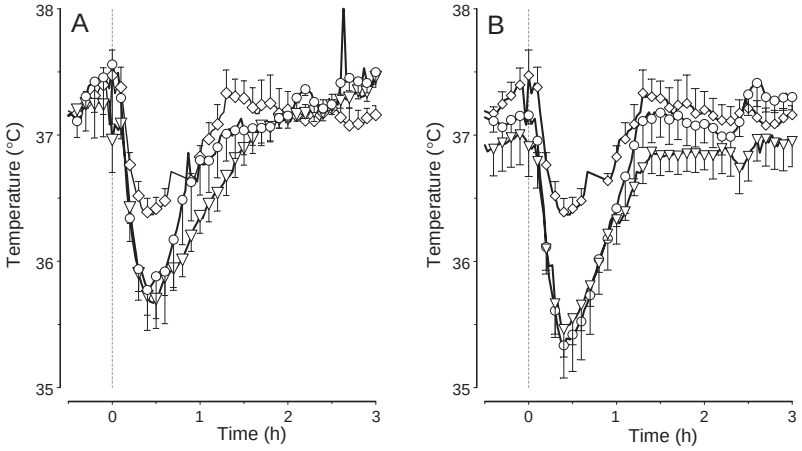

Fig. 8. Attenuation of tolerance to DPAT on day 3 by clomethiazole (A) and dizocilpine (B). Averaged (mean ± S.E.M., $n=5$–6) temperature responses upon s.c. injection of 0.3 μmol/kg R-8-OH-DPAT administered at $t=0$ (dotted line), which was preceded 48 h earlier by pretreatment of 3 μmol/kg DPAT (◇, E1), 300 μmol/kg clomethiazole (○, E5), or 300 μmol/kg clomethiazole + 3 μmol/kg DPAT (▽, E3). In the right panel, the pretreatment 48 h earlier was 3 μmol/kg DPAT (◇, E1), 3 μmol/kg MK (○, E8), or MK + DPAT (▽, E7).

zole completely attenuated the partial tolerance development to R-8-OH-DPAT. The temperature responses following an s.c. injection of 0.3 μmol/kg R-8-OH-DPAT 48 h after administration of 3 μmol/kg R-8-OH-DPAT (E1) or 3 μmol/kg dizocilpine together with 3 μmol/kg R-8-OH-DPAT (E7) or 3 μmol/kg dizocilpine (E8) are shown in Fig. 8B. Pretreatment with 3 μmol/kg dizocilpine completely attenuated the partial tolerance development to R-8-OH-DPAT, in the same way as clomethiazole. Furthermore, no cross-tolerance was observed between clomethiazole and R-8-OH-DPAT or dizocilpine and R-8-OH-DPAT. In addition, similar to the observations for clomethiazole, dizocilpine reduced the temperature response of R-8-OH-DPAT (data not shown). For each group, the temperatures 0.5 h after injection are compared and listed in Table 5.

3.8. Systemic exposure to test compounds

The exposure levels to clomethiazole, R-8-OH-DPAT and dizocilpine in single and combined injections were measured and shown in Fig. 9. An s.c. injection of 300 μmol/kg clomethiazole resulted in a maximum exposure level of 70 ± 15 μM. The maximum plasma concentrations resulting from 0.3 and 3 μmol/kg R-8-OH-DPAT were 60 ± 10 nM and 420 ± 70 nM, respectively. A dose of 3 μmol/kg dizocilpine resulted in a maximum plasma concentration of 240 ± 35 nM. The pharmacokinetic profiles for clomethiazole, R-8-OH-DPAT and dizocilpine were not altered in the presence of each other, confirming that no pharmacokinetic interaction was present. No exposure to muscimol, picrotoxin and bicuculline was obtained since the temperature measurements were part of another study. However, deduced from temperature and behavioral observations, it was considered that for all compounds, sufficient exposure was obtained.

4. Discussion

Clomethiazole induced an exposure-related decrease in body temperature that lasted between 6 and 18 h. During continuous s.c. administration, it was observed that despite steady-state plasma concentrations of clomethiazole, the hypothermic effect completely disappeared due to the development of tolerance. The finding that even a single s.c. injection of 300 μmol/kg clomethiazole in rats induced complete tolerance to a second injection of clomethiazole that lasted for 10 days and was still apparent after 32 days indicates that clomethiazole interferes with an unknown mechanism necessary for the development of the clomethiazole-induced hypothermia. It was confirmed in an additional experiment with an acidic saline injection as control that the tolerance was due to clomethiazole itself. Kalant and Khanna (1986) examined the development of tolerance to clomethiazole-induced hypothermia after chronic treatment of Wistar rats for up to 1 month, with daily intraperitoneal (i.p.) doses of 620 to 1000 μmol/kg clomethiazole. The authors concluded, however, that the tolerance that developed appeared to be due to altered disposition of the drug rather than to functional tolerance. The reason for the discrepant results obtained in this and our study is unclear. One explanation may be that in the study of Kalant and Khanna (1986), rats appear to have been used in an acute dose–response study of clomethiazole before being divided in two groups injected with clomethiazole and vehicle, respectively. If so, the control rats had also been injected with an initial dose of clomethiazole, which, according to our observations, is sufficient to evoke very long-lasting tolerance. Furthermore, the authors based their conclusion of altered disposition also on the fact that with an increased clomethiazole dose, only a small increase in blood concentrations was found. However, this nonlinear increase

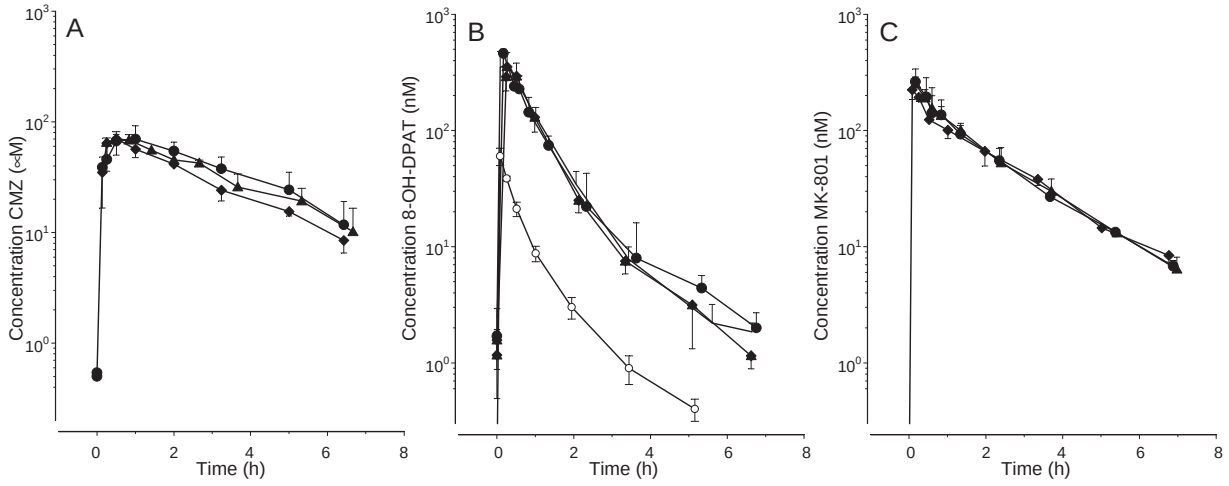

Fig. 9. Averaged (mean ± S.E.M., $n = 3$) plasma concentrations after s.c. injection of (A) 300 μmol/kg clomethiazole with pretreatment of vehicle (▲), 3 μmol/kg dizocilpine (♦), or 3 μmol/kg R-8-OH-DPAT (●). (B) 0.3 μmol/kg DPAT (○) and 3 μmol/kg DPAT with pretreatment of vehicle (▲), 3 μmol/kg MK-801 (♦), or 300 μmol/kg clomethiazole (●). C: 3 μmol/kg MK-801 with pretreatment of vehicle (♦), 3 μmol/kg DPAT (▲), or 300 μmol/kg clomethiazole (●).

in concentration can be expected at these dose levels, based on the nonlinear disposition of clomethiazole (see Fig. 1).

Because clomethiazole is known to activate $GABA_A$ receptor responses (Leeb-Lundberg et al., 1981; Harrison and Simmonds, 1983; Ogren, 1986; Hedlund and Ogren, 1987; Simmonds and Turner, 1987; Cross et al., 1989; Moody and Skolnick, 1989; Vincens et al., 1989; Zhong and Simmonds, 1997; Green, 1998), we examined whether $GABA_A$ receptor function is involved in the hypothermia and tolerance development. The results of the experiments performed suggest that this is not the case. First, the $GABA_A$ receptor agonist muscimol, which itself induced hypothermia, did not evoke any tolerance or cross-tolerance with clomethiazole. Furthermore, the $GABA_A$ receptor antagonist, bicuculline, and the noncompetitive $GABA_A$ receptor antagonist, picrotoxin, did not antagonize the hypothermic effect of clomethiazole or the tolerance development. By blocking the $GABA_A$ receptor–chloride channel complex, these antagonists induce convulsions and death. The doses used in our experiments were subconvulsant but evoked behavioral effects indicating central nervous effects. Muscimol-induced hypothermia could be blocked by pretreatment with similar doses of bicuculline and picrotoxin given i.p., even with a 30- to 60-min interval between pretreatment and muscimol injection (Zarrindast and Oveissi, 1988). Furthermore, the effect of clomethiazole on the tolerance of 8-PH-DPAT was also blocked by similar doses of bicuculline and picrotoxin given subcutaneously (Kelder and Ross, 2002). It is unlikely, therefore, that these doses were too low and too short-acting to antagonize the clomethiazole-induced hypothermia. Further experiments with $GABA_A$ receptor antagonists with a longer half-life, e.g., pentylenetetrazole, may be performed to evaluate the potential involvement of $GABA_A$ receptors in the clomethiazole-induced hypothermia and the development of tolerance. Nevertheless, the finding that muscimol is unable to induce hypothermic tolerance and cross-tolerance with clomethiazole indicates that the hypothermia induced by clomethiazole does not occur via interaction with the $GABA_A$ receptor complex. Furthermore, this indicates that the tolerance development does not seem to be due to hypothermia per se.

Rapid tolerance to hypothermia and motor impairment induced by ethanol, pentobarbital and chlordiazepoxide have been reported in rats (Khanna et al., 1991a,b, 1992). Pretreatment of the rats with the NMDA receptor antagonist, dizocilpine, inhibited this tolerance development (Khanna et al., 1991a, 1992). We therefore examined the effect of dizocilpine on the clomethiazole-induced hypothermia. Although dizocilpine almost completely inhibited the acute hypothermic effect of clomethiazole, it did not affect the tolerance development. This finding suggests that the mechanism of the clomethiazole-induced tolerance differs from that of ethanol and pentobarbital. Furthermore, it again shows that the tolerance develops independently of the hypothermia per se. Clomethiazole does not directly interact with NMDA or other glutamate receptors (Green et al., 1998; Empson et al., 2000). However, it does antagonize some functional responses mediated by glutamate receptors, e.g., NMDA receptor-mediated seizures and behavioral effects, probably by enhancing GABAergic transmission (Cross et al., 1993). A possible explanation of the inhibition by dizocilpine of the acute hypothermic effect of clomethiazole is that the clomethiazole-induced hypothermia is dependent on an intact NMDA receptor function. The pharmacokinetic analysis did not show any interaction between clomethiazole and dizocilpine that could explain the inhibition of the acute hypothermic effect of clomethiazole.

Rapid and long-lasting tolerance to the hypothermia induced by the $5\text{-}HT_{1A}$ receptor agonist, R-8-OH-DPAT, in rats has been reported (Renyi et al., 1992; Ross et al., 1992; Kelder and Ross, 2002). This tolerance was antagonized by NMDA receptor antagonists (Renyi et al., 1992; Ross et al., 1992) and compounds that facilitate $GABA_A$ receptor function (Kelder and Ross, 2002). The possible interaction between R-8-OH-DPAT and clomethiazole was therefore examined. Although the tolerance to R-8-OH-DPAT was less apparent in these experiments than that reported previously, significant tolerance to the hypothermic effect of R-8-OH-DPAT was found both after a bolus s.c. injection and after a continuous s.c. infusion via osmotic pumps. The dizocilpine sensitivity of the R-8-OH-DPAT-induced tolerance was also confirmed. The discrepancy in the extent of the tolerance obtained in the present and the previous experiments is most probably explained by the fact that the strain of the Sprague–Dawley rats (B&K) is less sensitive to the R-8-OH-DPAT-induced response than the ALAB Sprague–Dawley rats used in previous experiments (Kelder and Ross, 2001). The absence of any interaction between R-8-OH-DPAT and clomethiazole in the tolerance to clomethiazole seems to exclude the involvement of $5\text{-}HT_{1A}$ receptors in the development of the tolerance.

By excluding the involvement of $GABA_A$, NMDA or $5\text{-}HT_{1A}$ receptors, the question remains what causes the long-lasting tolerance to clomethiazole-induced hypothermia. The tolerance development occurred at low steady-state plasma concentrations (~8 μM) of clomethiazole, which are at least tenfold lower than the IC_{50} for [^{35}S]-TBPS binding, which is an indication of $GABA_A$ receptor modulation (Green, 1998). The potentially relevant action of clomethiazole reported so far has been the potent inhibition (IC_{50}=2 μM) of LPS- or IL-1β-inducible c-*fos* and c-*jun* expression and AP-1 activation by inhibition of p38 MAP kinase activity, which are involved in inflammatory processes and were suggested to play a role in the neuroprotective effects (Simi et al., 2000, 2002). It is known that IL-1β and LPS can play a role in thermogenesis (Mracek et al., 2004; Kenney et al., 2001). Furthermore, clomethiazole has been shown to specifically inhibit cytochrome *P*450-2E1 (CYP2E1, Eap et al., 1998; Simi and Ingelman-Sundberg, 1999). CYP2E1 is involved in the metabolism of many low-

molecular weight compounds, acetone, organic solvents, acetaminophen, toxins and carcinogens (Eap et al., 1998; Gebhardt et al., 1997) and can also be modulated by inflammatory cytokines like IL-1β (Hakkola et al., 2003). One may only speculate whether these processes are involved in the hypothermia and development of tolerance to clomethiazole and thus remain to be investigated.

In the experiment with R-8-OH-DPAT delivered via osmotic pumps, a significant rebound hyperthermic effect was shown when the pumps were removed after 24 h (Fig. 7). This rebound effect may be due to the existence of a counteracting mechanism when the tolerance to the R-8-OH-DPAT-induced hypothermia is established. In a study of the behavioral effects of the 5-HT_{1A} receptor agonists NAE-086 and R-8-OH-DPAT, it was found that when the tolerance to the 5-HT_{1A} receptor-mediated effects was established, the response to 5-HT_{2A} receptor-induced wet-dog shakes was sensitized (Renyi et al., 2001). 5-HT_{1A} and 5-HT_{2A} receptors seem to have opposite roles in the thermoregulation in rats (Gudelsky et al., 1986; Salmi and Ahlenius, 1998). A possible explanation of the rebound effect on the body temperature observed after withdrawal of R-8-OH-DPAT may therefore be the stimulation of 5-HT_{2A} receptors in the hypothalamus by endogenous 5-HT released from the serotonergic nerve terminals, when the inhibitory action on the nerve firing induced by stimulation of somatodendritic 5-HT_{1A} receptors disappeared and the tolerance to the postsynaptic 5-HT_{1A} receptors developed. Further experiments with 5-HT_{2A} receptor antagonists may answer this question.

In conclusion, rapid and long-lasting tolerance to the clomethiazole-induced hypothermia in rats is described in the present study. This tolerance does not seem to involve $GABA_A$ or 5-HT_{1A} receptor functions. The counteraction by dizocilpine of the clomethiazole-induced hypothermia suggests that glutamate via the NMDA receptor–Ca^{2+} channel complex may be involved in the decrease in the body temperature but not in the tolerance development.

Acknowledgements

The authors would like to thank Richard Green for critically reading the manuscript. We are also grateful to Marie Dahlgren and Johan Pettersson for the sample analysis of dizocilpine and R-8-OH-DPAT, and Lars B. Nilsson for his advice with the clomethiazole analysis.

References

Canini, F., Brejot, T., d'Aleo, P., Mercier, S., Bourdon, L., 2001. NMDA receptors are involved in dithiothreitol-induced hypothermia. Eur. J. Pharmacol. 426, 179–183.

Chaulk, D., Wells, J., Evans, S., Jackson, D., Corbett, D., 2003. Long-term effects of clomethiazole in a model of global ischemia. Exp. Neurol. 182, 476–482.

Cross, A.J., Stirling, J.M., Robinson, T.N., Bowen, D.M., Francis, P.T., Green, A.R., 1989. The modulation by chlormethiazole of the GABAA-receptor complex in rat brain. Br. J. Pharmacol. 98, 284–290.

Cross, A.J., Jones, J.A., Baldwin, H.A., Green, A.R., 1991. Neuroprotective activity of chlormethiazole following transient forebrain ischaemia in the gerbil. Br. J. Pharmacol. 104, 406–411.

Cross, A.J., Snape, M.F., Green, A.R., 1993. Chlormethiazole antagonises seizures induced by *N*-methyl-DL-aspartate without interacting with the NMDA receptor complex. Psychopharmacology (Berlin) 112, 403–406.

Eap, C.B., Schnyder, C., Besson, J., Savary, L., Buclin, T., 1998. Inhibition of CYP2E1 by chlormethiazole as measured by chlorzoxazone pharmacokinetics in patients with alcoholism and in healthy volunteers. Clin. Pharmacol. Ther. 64, 52–57.

Empson, R.M., Gee, V.J., Sheardown, M.J., Newberry, N.R., 2000. Chlormethiazole inhibits epileptiform activity by potentiating GABA(A) receptor function. Brain Res. 884, 31–34.

Evans, J.O., Feuerlein, W., Glatt, M.M., Kanowski, S., Scott, D.B., 1986. Chlormethiazole 25 years: recent developments and historical perspectives. Acta Psychiatr. Scand. 73 (Suppl. 327), 1–198.

Gebhardt, A.C., Lucas, D., Menez, J.F., Seitz, H.K., 1997. Chlormethiazole inhibition of cytochrome P450 2E1 as assessed by chlorzoxazone hydroxylation in humans. Hepatology 26, 957–961.

Green, A.R., 1998. Clomethiazole (Zendra) in acute ischemic stroke: basic pharmacology and biochemistry and clinical efficacy. Pharmacol. Ther. 80, 123–147.

Green, A.R., Murray, T.K., 1989. A simple intravenous infusion method in rodents for determining the potency of anticonvulsants acting through GABAergic mechanisms. J. Pharm. Pharmacol. 41, 879–880.

Green, A.R., Misra, A., Hewitt, K.E., Snape, M.F., Cross, A.J., 1998. An investigation of the possible interaction of clomethiazole with glutamate and ion channel sites as an explanation of its neuroprotective activity. Pharmacol. Toxicol. 83, 90–94.

Gudelsky, G.A., Koenig, J.I., Meltzer, H.Y., 1986. Thermoregulatory responses to serotonin (5-HT) receptor stimulation in the rat. Evidence for opposing roles of 5-HT2 and 5-HT1A receptors. Neuropharmacology 25, 1307–1313.

Hakkola, J., Hu, Y., Ingelman-Sundberg, M., 2003. Mechanisms of down-regulation of CYP2E1 expression by inflammatory cytokines in rat hepatoma cells. J. Pharmacol. Exp. Ther. 304, 1048–1054.

Harrison, N.L., Simmonds, M.A., 1983. Two distinct interactions of barbiturates and chlormethiazole with the GABAA receptor complex in rat cuneate nucleus in vitro. Br. J. Pharmacol. 80, 387–394.

Hedlund, B., Ogren, S.O., 1987. Chlormethiazole acts on chloride channels in cultured spinal cord neurons. Neurosci. Lett. 78, 217–221.

Kalant, H., Khanna, J.M., 1986. Metabolic and functional aspects of tolerance to chlormethiazole and cross-tolerance to ethanol in the rat. Acta Psychiatr. Scand., Suppl. 329, 45–53.

Kelder, D., Ross, S.B., 1993. Counteraction of the rapid tolerance to 8-OH-DPAT-induced corticosterone secretion in rats by activation of the GABAA receptor–chloride channel complex. Br. J. Pharmacol. 109, 207–212.

Kelder, D., Ross, S.B., 2001. Differential sensitivity to 8-hydroxy-2-(di-*n*-propylamino)tetralin-induced corticosterone secretion and hypothermia between Sprague–Dawley rats from different breeding colonies. Pharmacol. Toxicol. 89, 295–300.

Kelder, D., Ross, S.B., 2002. Counteraction of the rapid tolerance to 8-hydroxy-2-(di-*n*-propylamino)tetralin-induced hypothermia in rats by activation of the GABAA receptor–chloride channel complex. Pharmacol. Toxicol. 90, 14–19.

Kenney, M.J., Blecha, F., Morgan, D.A., Fels, R.J., 2001. Interleukin-1 beta alters brown adipose tissue but not renal sympathetic nerve responses to hypothermia. Am. J. Physiol.: Heart Circ. Physiol. 281, H2441–H2445.

Khanna, J.M., Kalant, H., Shah, G., Weiner, J., 1991. Rapid tolerance as an index of chronic tolerance. Pharmacol. Biochem. Behav. 38, 427–432.

Khanna, J.M., Wu, P.H., Weiner, J., Kalant, H., 1991. NMDA antagonist inhibits rapid tolerance to ethanol. Brain Res. Bull. 26, 643–645.

Khanna, J.M., Kalant, H., Weiner, J., Chau, A., Shah, G., 1992. Ketamine retards chronic but not acute tolerance to ethanol. Pharmacol. Biochem. Behav. 42, 347–350.

Leeb-Lundberg, F., Snowman, A., Olsen, R.W., 1981. Interaction of anticonvulsants with the barbiturate–benzodiazepine–GABA receptor complex. Eur. J. Pharmacol. 72, 125–129.

Moody, E.J., Skolnick, P., 1989. Chlormethiazole: neurochemical actions at the gamma-aminobutyric acid receptor complex. Eur. J. Pharmacol. 164, 153–158.

Mracek, T., Cannon, B., Houstek, J., 2004. IL-1 and LPS but not IL-6 inhibit differentiation and downregulate PPAR gamma in brown adipocytes. Cytokine 26, 9–15.

Ogren, S.O., 1986. Chlormethiazole-mode of action. Acta Psychiatr. Scand., Suppl. 329, 13–27.

Renyi, L., Moller, K.A., Ensler, K., Evenden, J., 1992. The non-competitive NMDA receptor antagonist (+)MK-801 counteracts the long-lasting attenuation of the hypothermic response induced by acute doses of 8-OH-DPAT in the rat. Neuropharmacology 31, 1265–1268.

Renyi, L., Evenden, J.L., Fowler, C.J., Jerning, E., Kelder, D., Lake-Bakaar, D., Larsson, L.G., Mohell, N., Sallemark, M., Ross, S.B., 2001. The pharmacological profile of (R)-3,4-dihydro-*N*-isopropyl-3-(*N*-isopropyl-*N*-propylamino)-2H-1-benzopyran-5-carboxamide, a selective 5-hydroxytryptamine(1A) receptor agonist. J. Pharmacol. Exp. Ther. 299, 883–893.

Ross, S.B., Renyi, L., Kelder, D., 1992. *N*-methyl-D-aspartate receptor antagonists counteract the long lasting 5-HT1A receptor-induced attenuation of postsynaptic responses in the rat in vivo. Naunyn-Schmiedeberg's Arch. Pharmacol. 346, 138–143.

Salmi, P., Ahlenius, S., 1998. Evidence for functional interactions between 5-HT1A and 5-HT2A receptors in rat thermoregulatory mechanisms. Pharmacol. Toxicol. 82, 122–127.

Shuaib, A., Mazagri, R., Ijaz, S., 1993. GABA agonist "muscimol" is neuroprotective in repetitive transient forebrain ischemia in gerbils. Exp. Neurol. 123, 284–288.

Simi, A., Ingelman-Sundberg, M., 1999. Post-translational inhibition of cytochrome P-450 2E1 expression by chlomethiazole in Fao hepatoma cells. J. Pharmacol. Exp. Ther. 289, 847–852.

Simi, A., Ingelman-Sundberg, M., Tindberg, N., 2000. Neuroprotective agent chlomethiazole attenuates c-fos, c-jun, and AP-1 activation through inhibition of p38 MAP kinase. J. Cereb. Blood Flow Metab. 20, 1077–1088.

Simi, A., Porsmyr-Palmertz, M., Hjerten, A., Ingelman-Sundberg, M., Tindberg, N., 2002. The neuroprotective agents chlomethiazole and SB203580 inhibit IL-1beta signalling but not its biosynthesis in rat cortical glial cells. J. Neurochem. 83, 727–737.

Simmonds, M.A., Turner, J.P., 1987. Potentiators of responses to activation of gamma-aminobutyric acid (GABAA) receptors. Neuropharmacology 26, 923–930.

Vincens, M., Enjalbert, A., Lloyd, K.G., Paillard, J.J., Thuret, F., Kordon, C., Lechat, P., 1989. Evidence that clomethiazole interacts with the macromolecular GABA A-receptor complex in the central nervous system and in the anterior pituitary gland. Naunyn-Schmiedeberg's Arch. Pharmacol. 339, 397–402.

Zarrindast, M.R., Oveissi, Y., 1988. GABAA and GABAB receptor sites involvement in rat thermoregulation. Gen. Pharmacol. 19, 223–226.

Zhong, Y., Simmonds, M.A., 1997. Interactions between loreclezole, chlormethiazole and pentobarbitone at GABA(A) receptors: functional and binding studies. Br. J. Pharmacol. 121, 1392–1396.