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N-Acetylcysteine ethyl ester (NACET): A novel lipophilic cell-permeable cysteine derivative with an unusual pharmacokinetic feature and remarkable antioxidant potential

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ABSTRACT

Recent large clinical trials failed to confirm the supposed beneficial effects of *N*-acetylcysteine (NAC) in preventing oxidative stress-related diseases. This may be due to its low bioavailability. We thought that esterification of the carboxyl group of NAC to produce *N*-acetylcysteine ethyl ester (NACET) would drastically increase the lipophilicity of NAC, thus greatly improving its pharmacokinetics.

In the present work, we report on representative chemical, pharmacological and anti-oxidant properties of NACET, especially in direct comparison with its congener NAC. We found that NACET is rapidly absorbed in rats after oral administration but reaches very low concentrations in plasma. This is due to a unique feature of NACET: it rapidly enters the cells where it is trapped being transformed into NAC and cysteine. After oral treatment, NACET (but not NAC) was able to increase significantly the glutathione content of most tissues examined, brain included, and to protect from paracetamol intoxication in the rat. NACET has also the unique feature to accumulate in human erythrocytes where it behaves as a potent protector against hydroperoxide-induced oxidative damage. Our study shows that being able to enter cells and to produce NAC and cysteine, NACET increases circulating hydrogen sulfide (H_2S), thus representing a good candidate for the oral use as an H_2S producer, with clear advantages over NAC.

NACET has the potential to substitute NAC as a mucolytic agent, as a paracetamol antidote and as a GSH-related antioxidant.

1. Introduction

Glutathione (γ-glutamylcysteinylglycine, GSH) has numerous physiological functions and plays a crucial role in the detoxification of xenobiotics, their metabolites and of reactive oxygen species (ROS). As GSH is synthesized from cysteine (Cys), the concentration of this amino acid in cells is of eminent importance and may be rate-limiting in GSH synthesis [1]. Therefore, supplementation of Cys or Cys-containing drugs, i.e., Cys pro-drugs, such as *N*-acetylcysteine (NAC), may be a feasible way to increase intracellular Cys levels thus boosting GSH synthesis. Actually, supplementation of Cys itself is not used to increase intracellular GSH concentration in vivo mainly for two reasons. In the first place, Cys is rapidly oxidized to the insoluble cystine (Cys-Cys), thus making difficult the preparation of stable Cys formulations. In the second place, it has been reported that Cys, at high concentrations, is toxic to cultured cells [2]. Therefore, the Cys content of parenteral formula is usually kept low [3].

NAC, the simplest Cys derivative, has been administered in a large number of reported studies [4,5]. NAC was introduced in the clinical practice more than 50 years ago. Its antimucolytic effect is assumed to be based on the reduction of the disulfide bridges of the proteins in the mucus. NAC therapy is also a standard clinical practice in acetaminophen (APAP; paracetamol) intoxication. Cytochrome P450-dependent oxidation of APAP yields the highly electrophilic and electrophilic intermediate *N*-acetyl-*p*-benzoquinone imine (NAPQI), which is able to bind to nucleophilic functionalities including free sulfhydryl (SH) groups. NAPQI is inactivated by GSH *S*-transferase-catalyzed conjugation with the cofactor GSH. In case of acetaminophen intoxication, hepatic GSH is depleted because high amounts of GSH are required, which cannot be satisfactorily provided by hepatocytes, erythrocytes and other cells of the intoxicated body [2,6]. In addition to these established main pharmacological actions of NAC, this thiol drug has a potent antioxidant activity.

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Oxidative stress is a condition determined by an imbalance between pro-oxidant production and antioxidant defense, and oxidative stress is generally thought to be related to several pathological conditions including atherosclerosis, diabetes, cancer and many other age-related diseases [7]. In theory, NAC itself should have a variety of protective antioxidant effects, however recent large clinical trials failed to confirm such beneficial effects [4 for a review]. A possible explanation for the antioxidant inefficacy of the drug NAC could be its low oral bioavailability, mainly due to its incomplete intestinal absorption and abundant pre-systemic metabolism to Cys and inorganic sulfate. It should also be considered that plasma is a rather pro-oxidizing milieu and, therefore, favors the oxidation of systemically available NAC to its symmetric disulfide (NAC–NAC) and to asymmetric mixed disulfides both with low- and high-molecular-mass thiols (e.g., Cys–NAC and protein–NAC, respectively). The bioavailability of authentic NAC is on the order of only 4–10% [2,8,9]. However, consideration of additional NAC forms including Cys, Cys–NAC and protein–NAC may finally yield considerably higher bioavailability values for NAC, as all of these forms may eventually contribute to enhance intracellular GSH levels. Burgunder et al. [10] reported that the AUC of total plasma cysteine and total plasma glutathione did not increase after oral administration of NAC (30 mg/kg) in healthy volunteers, although the AUC values of plasma NAC and Cys may markedly increase upon oral intake of NAC at the much lower dose of 10 mg/kg [8,11].

The relatively low oral bioavailability of NAC has stimulated the search for alternative pharmacologically favorable Cys pro-drugs. Several Cys and NAC derivatives have been prepared and tested to this aim [2 for a review]. As the carboxyl group of NAC is negatively charged at physiological pH values, NAC's ability to cross cell membranes is very limited. The newly prepared amide derivative of NAC (NAC-NH_2) has been shown to cross the blood–brain-barrier and to protect from oxidative injury in animal models and in cultured cells [12,13]. An interesting Cys product is represented by thiazolidines (e.g., 2-oxothiazolidine-4-carboxylic acid, OTC). OTC has shown a moderate effect in increasing GSH, but it has been reported to reverse endothelial dysfunction in patients with coronary artery disease [14]. We thought that esterification of the carboxyl group of NAC to produce *N*-acetylcysteine ethyl ester (NACET) would drastically increase the lipophilicity of NAC (Fig. 1), thus greatly improving its pharmacokinetics. In the present work, we report on representative chemical, pharmacological and antioxidant properties of NACET, especially in direct comparison with its congener NAC.

2. Materials and methods

2.1. Materials

Monobromobimane (mBrB) was obtained from Calbiochem (Milan, Italy). HPLC grade solvents were purchased from Mallinckrodt-Baker (Milan, Italy). NAC and all other reagents were obtained from Sigma–Aldrich (Milan, Italy) unless otherwise indicated.

The synthesis, purification and the mass spectrometry, ^{1}H NMR, infrared spectrometry, and polarimetry characterization of NACET ($C_7H_{13}NO_3S$, MW 191.2, mp 44.1–44.5 °C) were reported recently [15]. Briefly, NACET was prepared under argon atmosphere by *N*-acetylation of L-cysteine ethyl ester (Merck, Darmstadt, Germany) in dichloromethane with equimolar amounts of acetic anhydride (Merck, Darmstadt, Germany). HPLC analysis with UV (215 nm) absorbance detection of the isolated product revealed a chemical purity of >99% for NACET.

2.2. Animals

Sprague-Dawley rats (250–300 g) were purchased from Charles River (Calco, Milan, Italy). Rats were kept under controlled conditions (22–24 °C, relative humidity 40–50%, under a 12-h light/dark cycle) and fed ad libitum for 2–3 weeks before their use and during experiments. All animal manipulations were made in accordance with the European Community guidelines for the use of laboratory animals. The experiments were authorized by the Ethics Committee of the University of Siena.

2.3. Measurement of SH reactivity

The reactivity of the SH group of tested low molecular mass thiols (LMM-SH) was measured by reacting each compound (20 μM) with 0.2 mM 5,5′-dithiobis-(2-nitrobenzoic) acid (DTNB) in 0.1 M Na^+/K^+ phosphate buffer at pH 7.0. The reaction was analyzed spectrophotometrically by measuring absorbance at 412 nm wavelength according to the method of Ellman and Lysko [16]. Data were fitted to a simple exponential curve and the rate constants were calculated by Sigma Plot software (Jandel Scientific San Raphael, CA, USA).

2.4. Determination of distribution coefficients

For the determination of distribution coefficients in the octanol/water system, 1-mM solutions of NAC and NACET were prepared in 0.1 M Na^+/K^+ phosphate buffer, pH 7.4. Following partitioning between buffer and octanol (3 mL each, at room temperature 21 ± 1 °C) during 30 min on a horizontal shaker (1000 rotations/min) and phase separation by centrifugation (1 min, 10 000 × *g*), the aqueous phase was carefully separated and the NAC and NACET concentrations were measured by endpoint reaction, using an excess of DTNB [16]. The concentration of NAC and NACET in octanol was calculated by subtraction.

2.5. Pharmacokinetic studies

Rats were implanted either with a single or with a double valve (Model 415, 18 mm × 18 mm and model 620, 20 mm × 20 mm, Danuso Instruments, Milan, Italy). Jugular (single valve) or both jugular and femoral veins were cannulated (Dow Corning Silastic 0.51 mm i.d., 0.94 mm o.d., Biesterfeld, Milan, Italy) for drug administration (femoral) and blood collection (jugular) as previously described [17]. The valve was implanted under pentobarbital anesthesia (50 mg/kg) two days before the experiment and animals were allowed to freely move before and during the experiments.

Rats received orally 60 mg/kg NAC or an equivalent dosage of NACET in saline or intravenously 6 mg/kg NAC or an equivalent dosage of NACET. At certain time points blood aliquots (200 μL each) were collected through the valve connected to the jugular vein in tubes containing 5 μL of 50 mg/mL K_3EDTA and immediately centrifuged at 10 000 × *g* for 20 s in order to obtain plasma.

Analyses of total NAC (tNAC), total NACET (tNACET) and their metabolites Cys and GSH (i.e., the sum of reduced, low-molecular-mass disulfides and high-molecular-mass disulfides) in plasma were performed by treating 50 μL of samples with 2 mM dithiothreitol (DTT). After a 10-min incubation, samples were deproteinized by addition of 5% (w/v, final concentration) trichloroacetic acid (TCA), followed by centrifugation at 10 000 × *g* for 2 min. Then 40 μL of the supernatant were diluted with water (1:1, v/v), brought to pH ∼ 8.0 with 8 μL of 2 M Tris and then 5 μL of 40 mM mBrB dissolved in methanol were added. After a 10-min incubation in the dark, samples were acidified and analyzed by HPLC as previously described [18] with slight

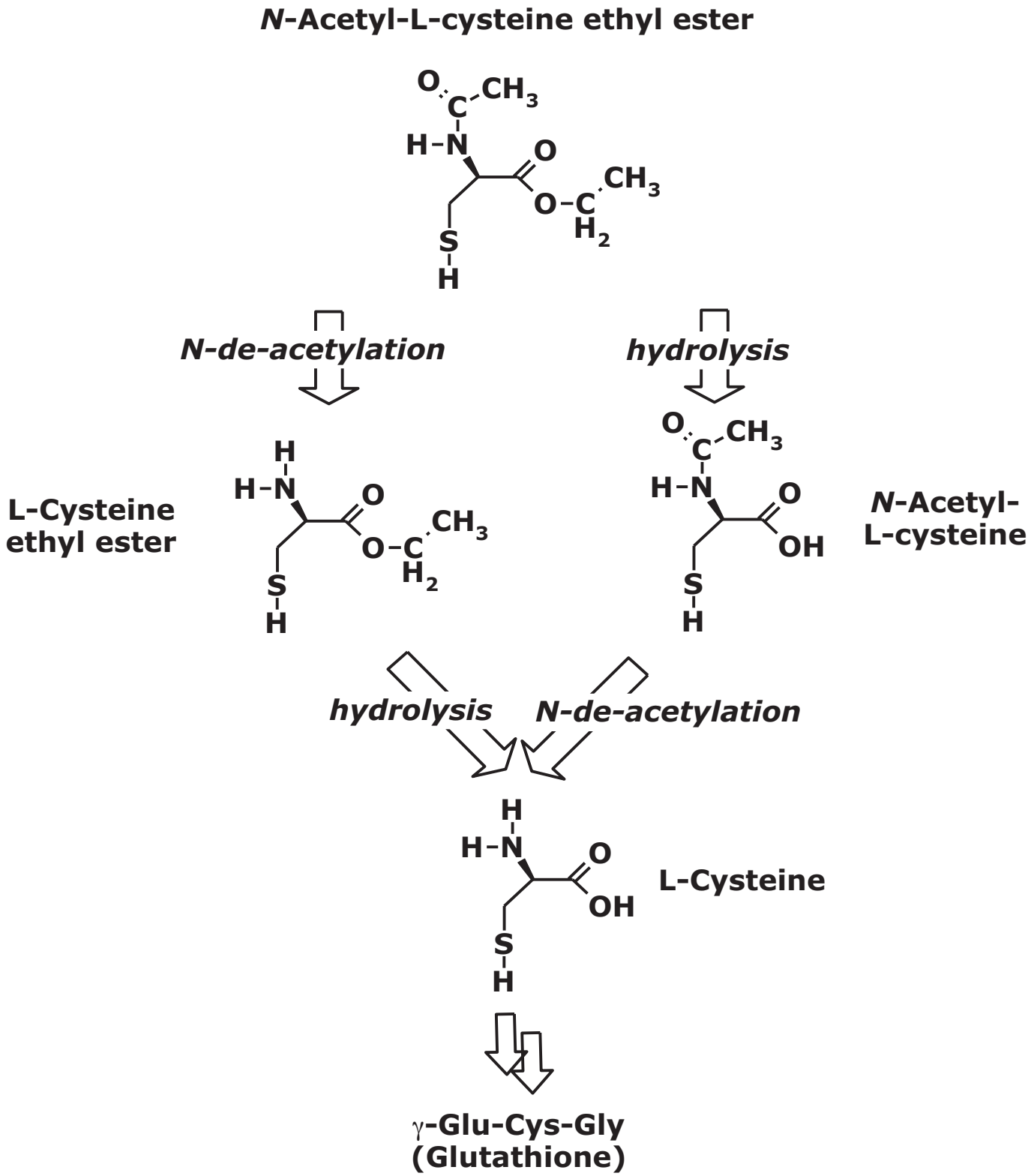

Fig. 1. Structural formulas of *N*-acetylcysteine ethyl ester (NACET), of its products of hydrolysis *N*-acetylcysteine (NAC) and *N*-de-acetylation cysteine ethyl ester (CET), and of the final cysteine (Cys), the common metabolite of *N*-de-acetylated NAC and hydrolyzed CET. The final reaction product Cys is supplied for the synthesis of the tripeptide glutathione (GSH).

modifications in order to measure NAC and NACET in addition. Specifically, HPLC separation was performed on a C18 column (Zorbax Eclipse XDB-C18 4.6 mm × 150 mm, 5 μm, Agilent Technologies, Milan, Italy). Elution conditions: solvent A = sodium acetate 0.25% (v/v), pH 3.09; solvent B = acetonitrile; 0–5 min: 94% solvent A/6% solvent B; 5–10 min linear gradient from 6% to 10% solvent B, 10–10.5 min linear gradient from 10% to 14% solvent B, 10.5–14.5 min 14% solvent B, 14.5–15 min linear gradient from 14% to 25% solvent B, 15–19 min linear gradient from 25% to 33% solvent B. A constant flow rate of 1.2 mL/min was applied. Detection was performed at 390 nm excitation and at 480 nm emission wavelength. In this HPLC system, NAC and NACET had a

retention time of 13.9 and 18.6 min, respectively. A calibration curve for each thiol was constructed in the 1–500 μM range. If out of range, sample was diluted with water and charged again. Standard solutions of Cys, γ-glutamylcysteine (γ-Glu-Cys), GSH, NAC and NACET were freshly prepared in water and titrated at the spectrophotometer by reaction with DTNB [16]. This procedure was shown to give equivalent results with respect to the standard addition method [18]. The identity of the peaks was confirmed by pre-treating samples with N-ethylmaleimide or by addition of thiols as depicted previously [19].

For the study of rapid drug distribution, animals were first anesthetized (pentobarbital, 50 mg/kg), treated by endovenous infusion (through penis vein) with a solution of ^{51}Cr-tagged rat red blood cells (RBCs) prepared according to Khor et al. [20] and after 2 min with 50 mg/kg NAC or the equivalent dosage of NACET. After 5 min from the last treatment rats were killed by decapitation, blood (about 2 mL) was collected in EDTA-containing tubes (50 μL of 50 mg/mL K_3EDTA in saline) and organs were rapidly removed, washed in NaCl 0.9% (w/v) and then immediately frozen in liquid nitrogen and stored at −80 °C until analysis. Aliquots of blood (0.4 mL) were rapidly centrifuged at 10 000 × *g* for 20 s to obtain plasma, which was stored immediately at −80 °C until analysis. Analyses of total LMM-SH in plasma were performed as above described. Erythrocytes were washed in Na^+/K^+ phosphate buffered saline containing 5 mM glucose and then hemolyzed by the addition of 100 volumes of 0.02 M Na^+/K^+ phosphate buffer and freshly measured for LMM-SH content by mBrB conjugation. Frozen tissues were homogenized (1:10, v/v) by Teflon/glass potter in 5% (w/v) TCA containing 1 mM of K_3EDTA and measured for LMM-SH content after mBrB conjugation. In both erythrocytes and tissues, LMM-SH were determined as previously described [18], with some modifications of HPLC separation conditions as above detailed. The determination of capillary blood volumes in organs was performed by measuring ^{51}Cr in an aliquot of tissue homogenate according to Khor et al. [20].

2.6. Isolated perfused liver experiments

Experiments with isolated perfused liver were carried out in the recirculation mode as described by Mancinelli et al. [21]. Briefly, after pentobarbital anesthesia (50 mg/kg) the common bile duct of rats was cannulated with polyethylene tubing (Dow Corning Silastic 0.4 mm i.d., 0.84 mm o.d. Biesterfeld, Milan, Italy), then animals were cannulated in situ by insertion of a 16-gauge cannula in the porta vein and a 20-gauge cannula in the upper cava vein, and then a single pass flux (25 mL/min) was started with a Hank's buffer. After clarification of the effluent, a recirculatory flux was started (150 mL total volume, flux 25 mL/min). Treatments were performed by adding 1 mM NAC or 1 mM NACET (final concentrations) to the perfusion medium. The viability of each perfused liver was assessed during each perfusion by monitoring oxygen consumption (>10 μmol/min), bile flow (>5 μL/min), percentage recovery of perfusate (>95%), and gross appearance of the organ. At the indicated times 0.5 mL aliquots of recirculating buffer were collected for analyses of total LMM-SH. Samples were centrifuged for 20 s at 10 000 × *g* and analyses of total LMM-SH were performed by HPLC after reduction of disulfides with DTT as described above. At the end of the perfusion, which lasted 90 min, the levels of LMM-SH were measured in liver homogenates as described above.

2.7. Evaluation of GSH replenishing effect

Animals were treated orally (by gavage) with 50 mg/kg twice a day of NAC or the equivalent amount of NACET for two weeks. The day after the last treatment in the morning (i.e., about 12 h from the last treatment) rat organs were rapidly removed under anesthesia (pentobarbital, 50 mg/kg), washed in NaCl 0.9% (w/v), immediately frozen in liquid nitrogen and stored at −80 °C until analysis. GSH, Cys and γ-Glu-Cys were measured in tissue homogenates by HPLC after conjugation with mBrB as above described.

2.8. Experiments on and measurement of hydrogen sulfide (H_2S)

A single valve (Model 515, 18 mm × 18 mm, Danuso Instruments, Milan, Italy) was implanted and jugular vein was cannulated for experiments. Drugs (50 mg/kg NAC or equivalent dosage of NACET) were orally administered by gavage twice, with an 8 h-interval time between the administrations. At the indicated times blood aliquots (200 μL each) were collected and plasma was obtained as above described. H_2S was measured in plasma by a modification of the methylene blue method [22] coupled with HPLC detection, as previously described [23]. Briefly, 20 μL of fresh plasma was added to 40 μL of 20% (w/v) TCA solution containing 1 mM K_3EDTA and 0.2 mM diethylenetriaminepentaacetic acid. After a 30-s centrifugation at 10 000 × *g*, 40 μL of the supernatant were added with 5 μL of *N*,*N*-dimethyl-*p*-phenylenediamine sulfate (DPD, 20 mM in 7.2 M HCl) and then 5 μL of $FeCl_3$ (30 mM in 1.2 M HCl). After a 20-min incubation in the dark, samples were analyzed by HPLC using a Zorbax Eclipse XDB-C18 column (4.6 mm × 150 mm, 5 μm, Agilent Technologies, Milan, Italy). The methylene blue formed from the reaction of H_2S with DPD in the presence of $FeCl_3$ was monitored at 667 nm wavelength by means of a diode array detector.

2.9. Study on protection from paracetamol overdose

Animals were randomly divided into four groups, each consisting of four animals. APAP was dissolved in saline and injected *i.p.* at the dose of 2 g/kg. Group 1-rats served as a control and received a single dose of 1 mL isotonic saline *i.p.* Group 2-rats were treated with a single dose of APAP. Group 3- and group 4-rats received APAP (2 g/kg) together with NAC (100 mg/kg) or the molar equivalent dosage of NACET, respectively. NAC and NACET dissolved in saline were administered orally 15 min before, and 2 and 4 h after APAP administration. After 6 h from APAP treatment, rats were anesthetized with pentobarbital (50 mg/kg), blood from abdominal aorta was collected and liver and kidney were removed for biochemical analyses.

Glutamic oxaloacetic transaminase/aspartate aminotransferase (GOT/AST), glutamic pyruvic transaminase/alanine aminotransferase (GPT/ALT) and lactate dehydrogenase (LDH) were measured in plasma by the Roche COBAS 6000 instrument (Roche Italia, Monza, Italy).

Tissues were homogenized (1:10, v/v) by Teflon/glass potter in 5% (w/v) TCA containing 1 mM of K_3EDTA. GSH was measured by HPLC after conjugation with mBrB as above described. Protein SH (PSH) groups were determined on aliquots of TCA deproteinized samples. Specifically, protein pellets were resuspended with a glass rod in 2% (w/v) sodium dodecyl sulfate containing 0.2 M phosphate buffer, pH 7.4, and put in a shaker until complete dissolution. Aliquot of samples were then diluted with the same buffer and reacted with DTNB as described above.

2.10. Experiments on human red blood cells

Human RBCs were obtained from the local blood bank, washed four times with buffered saline (20 mM phosphate buffer, pH 7.4) containing 6 mM glucose, resuspended at 50% (v/v) in the same buffer and utilized within the same day of collection, for the experiments. Erythrocytes were treated for 1 h with 1 mM NAC or

1 mM NACET at 37 °C. At certain time points aliquots of samples (0.3 mL) were collected and rapidly centrifuged at 10 000 × *g* for 20 s to separate extracellular milieu from RBCs. Both samples were then analyzed for NAC and NACET content by HPLC after conjugation with mBrB as described above. Experiments with *tert*-butyl hydroperoxide (*t*-BOOH) were performed by treating aliquots of 30% hematocrit RBCs with 1 mM NAC, 1 mM NACET or vehicle for 30 min at 37 °C. After a single rapid wash with buffered saline containing 6 mM glucose, RBCs were again resuspended at a 30% hematocrit and then were treated with 2.5 mM (final concentration) *t*-BOOH. At the indicated times, aliquots of samples (0.2 mL) were rapidly centrifuged to obtain a pellet of erythrocytes, which was used for both measurement of GSH and evaluation of oxidized hemoglobin after their hemolysis. GSH was measured by HPLC after incubation with mBrB as described above. The percentage of Hb^{3+} (methemoglobin) was calculated by spectral deconvolution by comparing the spectrum of the sample with standard spectra of reduced and oxidized Hb in the range 500–700 nm. The spectrum of reduced standard Hb was obtained by the addition of a few grains of sodium dithionite to RBC hemolysate and subsequent elution with PD-10 desalting columns equilibrated with 50 mM Na^+/K^+ phosphate buffer. The spectrum of oxidized Hb standard (met-Hb) was obtained by adding $K_3Fe(CN)_6$ in excess. Deconvolution was performed by fitting sample spectra by non-linear minimization (Sigma Plot program, version, Jandel Scientific, San Raphael, CA, USA). In all experiments no evidence of hemichrome formation was found.

An Agilent series 1100 HPLC (Agilent Technologies, Milan, Italy) equipped with diode array and a fluorescence detector was used for all determinations. All the spectrophotometric determinations were carried out by a Jasco V/550 instrument (Jasco Europe SRL, Modena, Italy).

2.11. Statistics and calculation of pharmacokinetic parameters

Data are expressed as means ± SD. Differences between means were evaluated using one-way analysis of variance (ANOVA). A value of $p < 0.05$ was considered statistically significant.

Pharmacokinetic parameters were calculated using Sigmaplot software (Jandel Scientific, San Raphael, CA, USA). The peak plasma concentration (C_{max}) and the time to the maximum plasma concentration (T_{max}) of NAC and NACET were noted directly from the plasma concentration vs time profiles. The area under the plasma concentration–time curve between 0 and ∞ ($AUC_{0-\infty}$) was calculated by using the linear trapezoidal rule method with extrapolation to infinite time. The clearance of drugs in isolated perfused liver experiments was calculated as $Dose/AUC_{0-\infty}$, where *Dose* is the dose of drug added to medium (expressed in mg) and $AUC_{0-\infty}$ is the area under the frequency output vs sampling time profile from time 0 to infinity, determined by the linear trapezoidal rule. The half-life ($t_{1/2}$) was calculated as $0.693/k_{el}$. The elimination rate constant (k_{el}) was determined by linear regression of the points of the natural logarithm concentration–time curves.

3. Results

3.1. Chemical properties of NACET

At room temperature, NACET (see Fig. 1) is a white powder with a melting point of about 44 °C [15]. NACET is freely soluble in water and organic solvents. The reactivity of the SH group of NACET was tested toward the typical thiol-reactant DTNB. As reported in Table 1, the free SH group of NACET reacted more rapidly with DTNB compared to other physiological LMM-SH. In particular, the reactivity of the SH group of NACET was about 10 times higher than that of NAC. We next determined the octanol/water distribution coefficients (log *D*) for NAC and NACET. The log *D* value was −5.4 for NAC and 0.85 for NACET. The almost six orders of magnitude higher log *D* of NACET impressively indicates that esterification of the carboxyl group of NAC by ethanol drastically increases the lipophilicity of the molecule. Interestingly, the esterification of NAC is associated with a considerable increase of the reactivity of the SH group toward the electrophile DTNB (Table 1).

Table 1
Reactivity of the sulfhydryl group of NACET and other low-molecular-mass thiols, $n = 3$.

Thiol	k_2 ($M^{-1} s^{-1}$)
Cysteine	$14.4 \pm 0.1 \times 10^5$
Glutathione	$5.70 \pm 0.2 \times 10^5$
N-Acetylcysteine ethylester	$17.5 \pm 0.3 \times 10^5$
Cysteamine	$14.1 \pm 0.0 \times 10^5$
N-Acetylcysteine	$1.77 \pm 0.05 \times 10^5$

3.2. Pharmacokinetics of NAC and NACET in the rat

Since both NAC and NACET can occur in plasma in their reduced (i.e., with the free –SH group) and oxidized forms (i.e., low-molecular-mass disulfides and mixed disulfides with proteins), the sum of all these forms, i.e., tNAC and tNACET, was considered in the pharmacokinetic study. Oral administration of NAC or NACET at equivalent dosages to rats (Fig. 2A) yielded comparable C_{max} values (69 ± 10 μM vs 96 ± 15 μM) but different T_{max} values (10 min vs 120 min). The plasma concentration–time profile of tNAC and tNACET upon intravenous administration of equivalent dosages to rats is shown in Fig. 2B. tNACET reached relatively low concentrations in plasma (C_{max} = 75 ± 12 μM) and disappeared quite rapidly from the plasma compartment ($t_{1/2}$ = 0.36 ± 0.06 h). Conversely, NAC infusion led to very high levels of tNAC in the plasma compartment (C_{max} = 1250 ± 220 μM) and tNAC disappeared slowly from the plasma compartment ($t_{1/2}$ = 4.35 ± 0.57 h). From the respective AUCs obtained and the dosages used, the oral bioavailability was determined to be only 4.8 ± 1.2% for NAC but 58.5 ± 8.8% for NACET. In the same experiments, we also measured the AUC values of the main metabolites Cys and GSH. However, given the relatively high physiological plasma concentrations of both Cys and GSH it was difficult to evidence reliable differences between NAC and NACET with respect to these compounds. In fact, only AUC for Cys (within 0–20 h) showed a tendency to be higher ($p = 0.043$, not shown) from oral treatment with NACET compared to NAC.

The data of Fig. 2 together with the high lipophilicity of NACET suggest that NACET is likely to be enriched within cells. To verify this possibility, rats were intravenously injected with NACET, NAC or vehicle, after 5 min several organs were rapidly removed, blood samples were collected and the reduced and oxidized forms of NACET, NAC, Cys and GSH were measured in plasma, RBCs and tissue homogenate (Table 2). Typical chromatograms obtained for thiol analyses are shown in Fig. 3. Upon NACET administration, most of the analyzed organs contained variable amounts of NACET but large amounts of NAC and Cys. It should be noted that in some blood-rich tissues (e.g., lung and heart) NAC may also derive from contaminating plasma. However, the raise in the Cys content is unequivocal since it mainly originates from intracellular sources. After correction for contaminating blood (Table 2, values reported in brackets) it is evident that intracellular concentration of NAC in all analyzed organs is largely higher in rats treated with NACET with respect to animals treated with NAC itself. The lack of increases in GSH levels is probably due to the high intracellular GSH concentrations, as well as due to the very short time following the drug administration. In most of the analyzed tissues, the increase in Cys in the animals treated with NACET is much more evident compared to the rats treated with NAC. It is worth

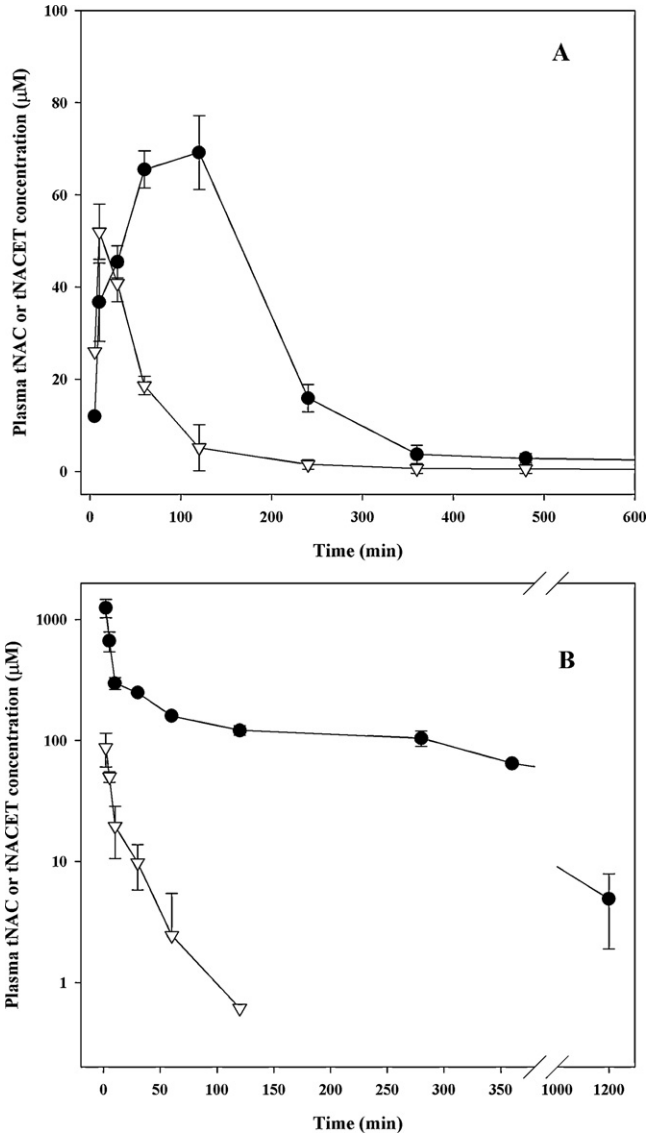

Fig. 2. Plasma total concentrations of NAC (tNAC, circles) and NACET (tNACET, triangles) after oral administration of 60 mg/kg NAC or the equivalent dosage of NACET (A) or after intravenous administration of 6 mg/kg NAC or the equivalent dosage of NACET (B) to rats (each $n = 4$). Data are the mean ± SD. At the indicated time points blood was taken and the concentration of total NAC (i.e., the sum of the free –SH form, the low-molecular-mass disulfide and the mixed disulfide with proteins) and of total NACET (i.e., the sum of the free SH form, the low-molecular-mass disulfide and the mixed disulfide with proteins) were measured. The first blood sample was taken 5 min after administration of the drugs.

mentioning that only NACET administration resulted in increased levels of NAC and Cys in rat brain. These data strongly suggest that NACET is able to rapidly enter inside the tissue cells, where it is converted to NAC, which is subsequently de-acetylated to Cys. NAC and Cys are supposed to be slowly released from the cells into the blood.

We performed additional experiments on isolated perfused rat liver (Fig. 4). The measured total levels of the drugs (i.e., the sum of the reduced form, the low-molecular-mass disulfide and the mixed disulfide with proteins for both NAC and NACET) in the medium during the perfusion clearly indicate that NACET but not NAC rapidly enters hepatocytes (Fig. 4A). Clearance values resulted to be only 0.62 ± 0.09 mL/min for NAC but 11.8 ± 0.6 mL/min for NACET. Total NACET concentration decreased rapidly and total Cys concentration rose concomitantly in the circulating medium (Fig. 4B), whereas perfusion of the liver with NAC resulted in very low

Table 2
NAC and NACET metabolites in rat tissues after a 5-min infusion either with 50 mg/kg NAC or with an equimolar dosage of NACET. Data (mean ± SD) are expressed as μM, $n = 4$ for each treatment.

Treatment	Cys	GSH	NAC	NACET
Plasma[a]				
NACET	276 ± 29**,[b]	25.7 ± 0.8	470 ± 26	538 ± 34
NAC	237 ± 39	22.9 ± 3.5	7840 ± 130	–
Vehicle	196 ± 8	28.4 ± 1.8	–	–
RBCs				
NACET	15.4 ± 0.9**,§§	2490 ± 100	431 ± 13§§	45.8 ± 5.3
NAC	6.47 ± 1.21	2300 ± 180	33 ± 19	–
Vehicle	5.03 ±0.38	2340 ± 150	–	–
Liver				
NACET	840 ± 102**,§§	8120 ± 380	297 ± 13 (277)[c]	24.1 ± 3.9
NAC	280 ± 27*	7820 ± 580	98.8 ± 11.5 (neg)	–
Vehicle	119 ± 15	7780 ± 390	–	–
Kidney				
NACET	398 ± 52	2960 ± 350	230 ± 13 (215)	25.9 ± 4.4
NAC	517 ± 87	2760 ± 180	250 ± 17 (51)	–
Vehicle	440 ± 35	2670 ± 80	–	–
Lung				
NACET	407 ± 30**,§§	2370 ± 140	229 ± 16 (208)	77.0 ± 11.0
NAC	124 ± 23**	2490 ± 230	318 ± 37 (neg)	–
Vehicle	65.8 ± 9.0	1990 ± 260	–	–
Heart				
NACET	288 ± 31**,§§	1950 ± 170	104 ± 11 (92)	40.4 ± 6.3
NAC	116 ± 7**	1880 ± 210	172 ± 14 (neg)	–
Vehicle	21.1 ± 5.6	1760 ± 190	–	–
Brain				
NACET	338 ± 23**,§§	2020 ± 70	233 ± 12 (232)	21.8 ± 2.5
NAC	51.7 ± 6.7	2070 ± 140	25.4 ± 3.8 (neg)	–
Vehicle	44.7 ± 6.5	2020 ± 120	–	–

[a] Values in plasma refer to the total amount (i.e., the sum of the reduced form, the low-molecular-mass disulfide form and the protein mixed disulfide form) of each low-molecular-mass thiol.
[b] $^{*}p < 0.05$ vs vehicle; $^{**}p < 0.01$ vs vehicle; $^{§§}p < 0.01$ vs NAC.
[c] Values in brackets are corrected by subtracting the NAC deriving from blood, "neg" means that the result of the subtraction is negative.

extracellular Cys concentrations, suggesting that only a very minor fraction of NAC is able to reach the interior of hepatocytes. This finding contradicts the general belief that NAC possesses a high hepatic fist-pass metabolism [9].

Again, the isolated perfused liver experiments indicate that NACET readily enters the cells where it is rapidly metabolized to NAC and Cys. Since liver perfusion with NACET was associated with low NAC levels but high Cys concentrations, we can reasonably conclude that NAC formed within hepatocytes is not exported but mainly remains therein (unless transformed into Cys). It is of note that the levels of LMM-SH measured in the liver tissue at the end of perfusion reveal consistently higher values of NAC and Cys in NACET-treated liver compared to the liver perfused with NAC (Table 3).

3.3. Effects on GSH levels upon chronic treatment with NAC or NACET

Rats were treated per os twice a day with NAC, NACET or vehicle with relatively high drug doses (50 mg/kg of NAC or an equivalent dosage of NACET). After 2 weeks a significant increase of GSH was obtained in some tissues, namely brain, liver, kidney, testis and heart only for NACET, whereas NAC was unable to elicit the same effect (Table 4). We also measured the levels of two prominent GSH precursors, namely Cys and γ-Glu-Cys in the same organs. Cys did not increase in the analyzed organs, with the exception of the brain, whereas γ-Glu-Cys was higher in some tissues with increased GSH content upon NACET treatment. Of particular interest is the finding that the brain was the organ in which not

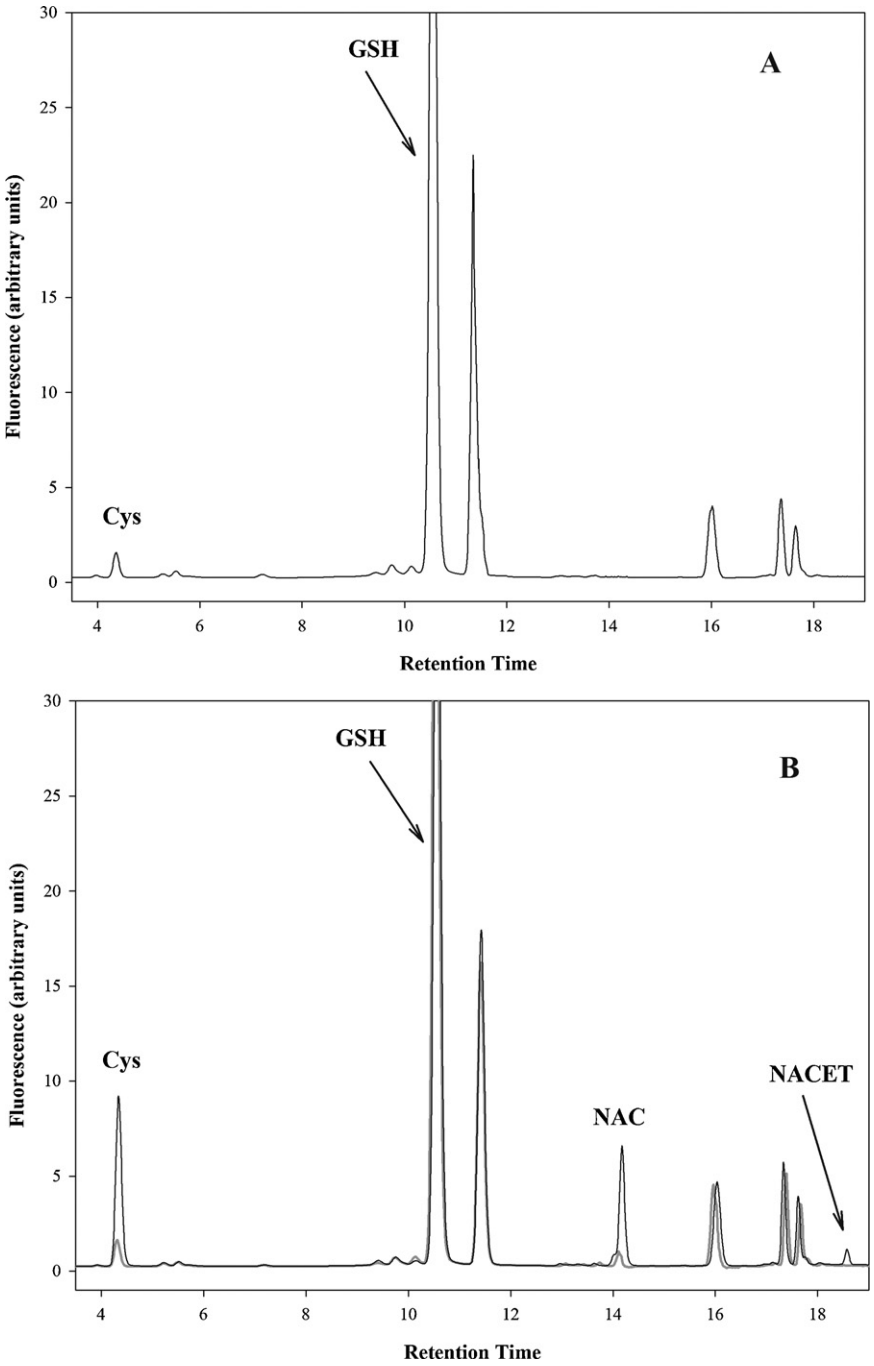

Fig. 3. Typical HPLC chromatograms for low molecular mass thiol analyses in rat brain. Rats were treated by endovenous infusion either with 50 mg/kg NAC (Panel B, gray tracing) or equimolar NACET (Panel B, black tracing) and after 5′ from treatment blood and several organs were rapidly collected and analyzed for the content of the parent drugs and their main metabolites. Control animals were treated with the vehicle (Panel A). Low molecular mass thiols were revealed after conjugation with the fluorescent label mBrB as described in Section 2.

only the GSH content but also the content of its precursors Cys and γ-Glu-Cys were significantly increased only in NACET-treated animals.

3.4. Effects on H_2S production

It has been recently reported that H_2S can be generated by the action of two enzymes of the transsulfuration pathway, i.e., cystathionine β-synthase (CBS) and cystathionine γ-lyase (CSE). Produced H_2S can have several effects and most of them seem to be regulatory and protective [24]. H_2S production from cells, in vivo, can be stimulated primarily by increasing cellular stores of Cys that can function as substrate of CSE. For this purpose both Cys and NAC have been tested [25]. We compared circulating levels of H_2S after oral administration of two equivalent doses of NAC and NACET given twice within at 8-h interval time between. Fig. 5 shows that plasma H_2S concentration increased immediately after NACET administration but not upon NAC administration. Although the pathways leading to H_2S formation are incompletely understood, our results suggest that only NACET is able to reach the cells at an appreciable extent that allows increased production of the gaseous molecule H_2S, which in turn spreads into the bloodstream.

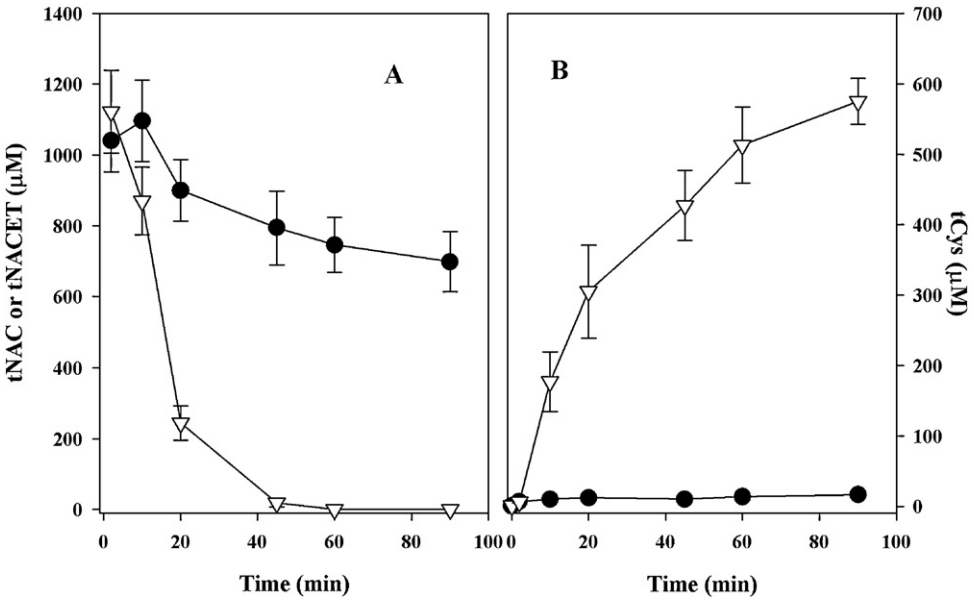

Fig. 4. Levels of total LMM-SH in medium from isolated perfused rat liver. Rat livers were perfused in the recirculation mode with Hank's buffer containing 1 mM NAC or 1 mM NACET. (Panel A) Concentration of total NAC (tNAC, i.e., the sum of the free –SH form, the low-molecular-mass disulfide and the mixed disulfide of NAC with proteins) (circles) and total NACET (tNACET, i.e., the sum of the free –SH form, the low-molecular-mass disulfide and the mixed disulfide of NACET with proteins) (triangles) in the recirculating buffer over the perfusion time. (Panel B) Concentration of total Cys (tCys, i.e. the sum of the free –SH form, the low-molecular-mass disulfide and the mixed disulfide of Cys with proteins) in the recirculating buffer over the perfusion time in isolated liver perfused with NAC (circles) or NACET (triangles). Data are the mean ± SD, $n = 4$ for each treatment.

3.5. Protection from paracetamol acute poisoning

One of the main established clinical usages of NAC is to treat paracetamol overdose, a life threatening condition. In the paracetamol overdose setting, a large amount of acetaminophen is metabolized by cytochrome P450 (CYP) to form NAPQI, which causes hepatic centrilobular necrosis, via a not fully clarified molecular mechanism [26]. It has been recently suggested that NAPQI reacts with free thiol groups of proteins and in turn leads to a mitochondrial damage and nuclear DNA fragmentation [27]. As NAPQI detoxification proceeds via GSH conjugation, GSH becomes depleted in paracetamol overdose. The utility of NACET to prevent paracetamol-induced toxicity was tested in acute poisoning experiments. After administration of 2 g/kg of APAP to rats it was evident that the thiol content of the main target organs (i.e., liver and kidney) was significantly higher in NACET group compared to the NAC and APAP groups (Table 5). Conversely, indicators of liver damage, namely GOT/AST, GPT/ALT and LDH, were consistently lower in the NACET group. These experiments suggest that NACET may more powerfully protect these tissues from paracetamol-induced damage than NAC.

Table 3
Levels of low-molecular-mass thiols in isolated perfused rat livers after a 90-min infusion with 1 mM NAC or NACET. Data (mean ± SD) are expressed as μM, $n = 4$ for each treatment.

Treatment	Cys	GSH	NAC	NACET
NACET	340 ± 66**,[a]	7280 ± 560	172 ± 9**	26.3 ± 4.2
NAC	101 ± 46	7030 ± 350	37.3 ± 1.2	–

[a] **$p < 0.01$ vs NAC perfusion.

3.6. Metabolic fate of NAC and NACET in human red blood cells

Washed human RBC suspensions (50%, v/v) were incubated for 1 h in the presence of NAC or NACET and the concentration of the thiols were measured both in the RBCs and in the extracellular milieu at the indicated times. NACET disappeared rapidly from the extracellular space, whereas extracellular NAC concentration remained relatively stable, i.e., only a 10% decrease was observed

Table 4
Effect of NACET and NAC (50 mg/kg twice a day per os for two weeks) on the levels of glutathione and its precursors cysteine and γ-glutamyl-cysteine. Data (mean ± SD) are expressed as μM, $n = 5$ for each treatment.

Treatment	Liver	Kidney	Lung	Heart	Spleen	Testis	Brain
Glutathione							
NACET	8150 ± 520*,[a]	3690 ± 170**,§§	2420 ± 160	2060 ± 36**	3420 ± 200	4960 ± 44**,§§	2130 ± 96*
NAC	7270 ± 1020	2960 ± 95	2220 ± 6	1820 ± 93	3450 ± 70	4160 ± 350	1920 ± 80
Vehicle	6980 ± 250	3060 ± 250	2350 ± 390	1650 ± 200	3500 ± 520	4100 ± 660	1810 ± 100
Cysteine							
NACET	84.2 ± 8.8	1000 ± 30**,§§	60.2 ± 14.0	30.7 ± 3.4	322 ± 48	114 ± 28	97.4 ± 5.1**,§§
NAC	78.0 ± 2.2	852 ± 11**	55.4 ± 15.2	28.7 ± 9.0	371 ± 18	88.5 ± 16.7	72.2 ± 5.3
Vehicle	78.2 ± 51.0	499 ± 139	54.6 ± 26.8	27.5 ± 3.6	406 ± 78	116 ± 22	65.3 ± 1.9
γ-Glutamyl-cysteine							
NACET	11.4 ± 0.9**	26.5 ± 3.4*	5.10 ± 1.72	7.82 ± 1.13	13.5 ± 0.2	14.0 ± 1.0**,§§	6.31 ± 1.02*
NAC	10.1 ± 3.2	21.7 ± 1.5	4.13 ± 1.17	6.20 ± 0.59	19.8 ± 1.8*	9.92 ± 0.68	4.69 ± 0.00
Vehicle	7.32 ± 2.20	20.0 ± 2.4	4.25 ± 0.85	5.21 ± 1.37	13.5 ± 0.2	8.00 ± 2.10	4.70 ± 0.30

[a] *$p < 0.05$ vs vehicle; **$p < 0.01$ vs vehicle; §§$p < 0.01$ vs NAC.

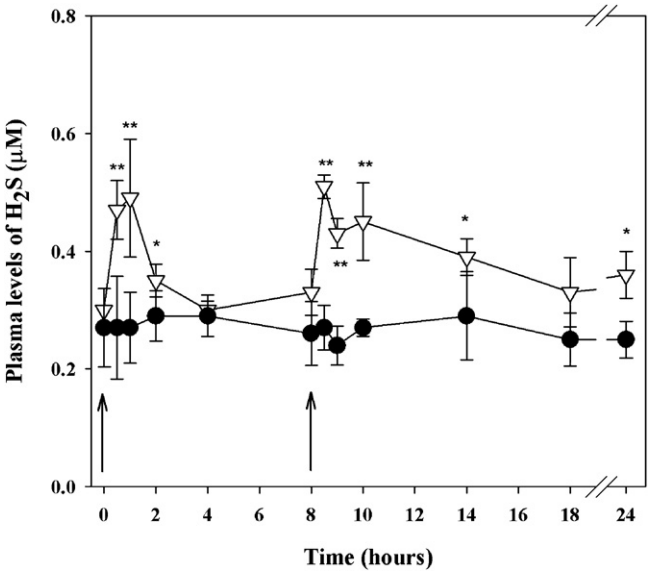

Fig. 5. Levels of hydrogen sulfide (H_2S) in plasma of rats upon NAC or NACET administration. NAC (50 mg/kg, circles) or NACET (an equivalent dose, triangles) were orally administered twice within an 8-h interval time between (at 0 and 8 h, as indicated by the arrows). At the indicated times blood aliquots were collected and plasma was analyzed for H_2S. Data are the mean ± SD, $n = 4$ for each treatment. $^{*}p < 0.05$ vs NACET; $^{**}p < 0.01$ vs NACET.

within 1 h (Fig. 6A). It is remarkable that the rapid decrease in extracellular NACET was not due to its oxidation to its disulfide, but to its entrance into the RBCs (Fig. 6B), where it was converted to NAC. Obviously, NACET-derived NAC in the RBCs cannot be exported but it is trapped in this compartment, whereas conversely extracellular NAC only very slowly enters RBCs.

RBCs pre-treated with NAC or NACET were challenged with the oxidizing agent *t*-BOOH. Treatment with *t*-BOOH led to a rapid GSH oxidation in both NAC and NACET pre-treated RBCs, but the recovery of initial intracellular GSH values was more rapid in NACET pre-treated RBCs (not shown). Importantly, the time course of hemoglobin oxidation was evidently much lower in NACET-protected RBCs. Eventually, we observed that only in NACET-protected RBCs the values of ferric hemoglobin tended to return to initial values after *t*-BOOH-induced oxidation (Fig. 7).

4. Discussion

The widespread concept on GSH is that this molecule, being an antioxidant and being involved in detoxification reactions, is fundamental for health maintenance. Under normal conditions, GSH is oxidized to glutathione disulfide (GSSG), which, in turn, is reduced back to GSH by glutathione reductase using NADPH as a cofactor [28]. If oxidative stress is severe, GSSG accumulates in cells and this has two important consequences: (i) protein *S*-glutathionylation occurs, which may result in activation/inactivation of regulatory pathways [29] and (ii) loss of intracellular GSH due to export of GSSG and its extracellular hydrolysis. Loss of intracellular GSH may also occur due to conjugation of GSH with electrophiles via the SH group of the cysteine moiety to form stable GSH thioethers, which are further metabolized via the mercapturic acid pathway [30]. Both these processes are associated with irreversible loss of intracellular GSH. Actually, GSH oxidation and consumption in biotransformation results in loss of Cys because GSH-derived glycine and glutamate are recycled. GSH is synthesized intracellularly by the action of two enzymes, i.e.,

Table 5
Effects of NAC and NACET on several damage biomarkers in paracetamol (acetaminophen, APAP) overdose in the rat. Data are the mean ± SD. $n = 4$ for each treatment.

Group	Plasma			Liver		Kidney	
	AST (U/L)	ALT (U/L)	LDH (U/L)	GSH (μmol/g tissue)	PSH (μmol/g tissue)	GSH (μmol/g tissue)	PSH (μmol/g tissue)
Control	70. 2 ± 3.0	78.4 ± 5.9	743 ± 69	7.32 ± 0.13	85.4 ± 6.2	2.87 ± 0.13	60.1 ± 3.2
Control + APAP	756 ± 8	116 ± 21	4800 ± 370	2.19 ± 0.57	43.7 ± 5.9	1.74 ± 0.21	38.8 ± 1.5
NAC + APAP	642 ± 36^{**,a}	90.0 ± 13.1	4100 ± 220**	2.33 ± 0.41	52.6 ± 2.2*	1.82 ± 0.11	42.0 ± 4.0
NACET + APAP	457 ± 19**,§§	68.1 ± 4.8**,§	3460 ± 110**,§§	2.78 ± 0.22**,§§	68.3 ± 3.6**,§§	2.38 ± 0.05**,§	52.2 ± 1.7**,§§

[a] $^{*}p < 0.05$ vs vehicle; $^{**}p < 0.01$ vs vehicle: $^{§}p < 0.05$ vs NAC; $^{§§}p < 0.001$ vs NAC.

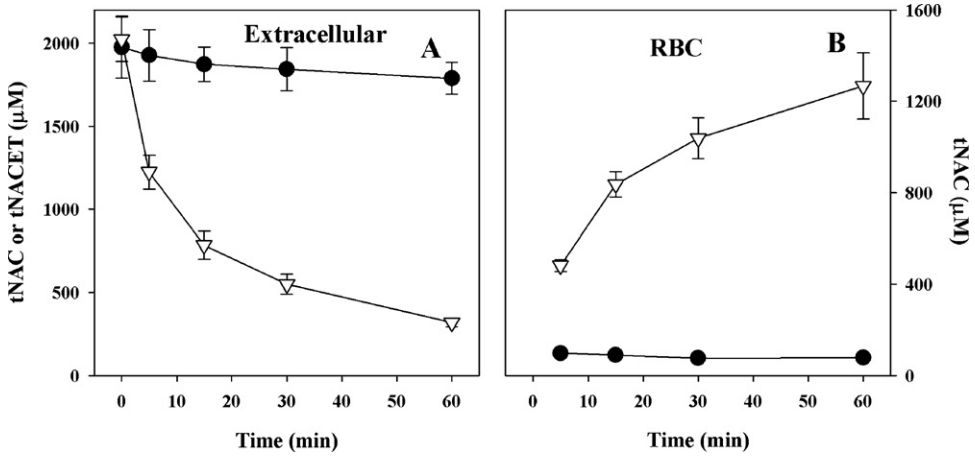

Fig. 6. NAC or NACET fate in human erythrocytes. A 50% suspension of human RBCs in 20 mM phosphate buffer, pH 7.4, containing 6 mM glucose, was treated with NAC or NACET (each 1 mM with respect to the whole sample volume). At the indicated times aliquots were taken, extracellular milieu was separated from RBCs, and the extracellular and intracellular total concentration of NAC (i.e., the sum of the free –SH form, the low-molecular-mass disulfide and the mixed disulfide of NAC with proteins, tNAC) and total concentration of NACET (i.e., the sum of the free –SH form, the low-molecular-mass disulfide and the mixed disulfide of NACET with proteins, tNACET) was measured. (Panel A) Levels of tNAC (circles) or tNACET (triangles) in the extracellular milieu. (Panel B) Levels of tNAC from treatment with NAC (circles) or from treatment with NACET (triangles) in RBCs. Data are the mean ± SD, $n = 5$ for each treatment.

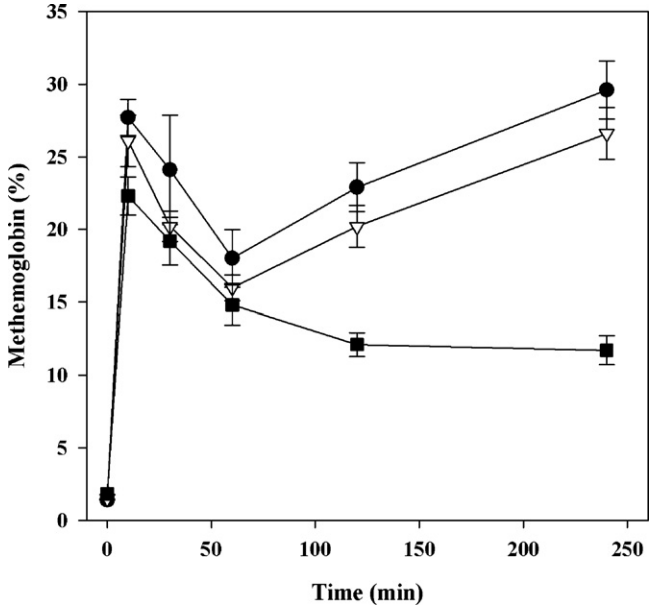

Fig. 7. Formation of methemoglobin in human erythrocytes treated with *t*-BOOH. A 50% suspension of human RBCs was pre-treated with 1 mM NAC (triangles), NACET (squares) or vehicle (circles) and then with 2.5 mM *t*-BOOH. At the indicated times sample aliquots were taken and obtained RBCs were analyzed for methemoglobin by spectrophotometry. Data are the mean ± SD, $n = 5$ for each treatment.

γ-glutamylcysteine synthase (γGCS) and glutathione synthase (GS). GSH biosynthesis is regulated by GSH feedback inhibition of γGCs and Cys availability. Even if some aspects of GSH biosynthesis have not been fully elucidated (e.g., the redox regulation of γGCs activity), the availability of Cys in cells synthesizing GSH is a crucial rate-limiting step [1].

Several different methods have been proposed for increasing tissue levels of Cys thus far. They are based on the use of synthetic drugs, notably NAC in humans, or on natural products, largely in animal models. While toxicological safety is not a concern, the efficacy of cysteine supplements remains questioned in many topics. NAC, in high doses, is routinely used as an antidote in paracetamol intoxication. On the other hand, as mucolytic agent, NAC is rated as ineffective for example in the UK [4]. Systematic reviews and meta-analyses suggested that prolonged treatment with NAC is inefficacious in chronic obstructive pulmonary disease [31]. In a large intervention trial on cancer recurrence, NAC was found to be ineffective too [32]. NAC infusion has been widely used in acute hepatic failure but convincing evidence of NAC's benefit is lacking [33]. Initially, NAC has been reported to be effective in preventing radio contrast-induced nephropathy [34], but subsequently highly inconsistent results have been reported. More recently, a large study disproved the pharmacological efficacy of NAC in radio contrast-induced nephropathy [35].

Orally administered NAC has been reported to be rapidly absorbed but also to undergo extended first-pass hepatic metabolism thus resulting in low bioavailability [36]. At first, this has been thought to be of minor importance because the effects of NAC are supposed to be exerted either by NAC itself or by its metabolites, notably Cys and GSH. However, abundant metabolism of NAC and Cys to inorganic sulfate and taurine needs also to be considered. The fact that NAC is not only de-acetylated to Cys, but it is also rapidly oxidized in plasma to its symmetric disulfide NAC–NAC and to mixed disulfides with LMM-SH and high molecular mass thiols, which could be considered as silent Cys stores, raised the question of whether the calculation of the bioavailability of NAC should be based on plasma NAC concentrations alone, or whether NAC and all of its forms circulating in blood and its metabolites should be rather considered. On the basis of circulating NAC, our results confirm that the oral bioavailability of NAC is below 5% (Fig. 2) [2,9]. On the same basis, our results indicate that the newly synthesized ethyl ester of NAC, i.e., NACET, has a much higher bioavailability (around 60%) than NAC, presumably because of a high absorption rate due to its lipophilicity and missing electrical charge at acidic and neutral pH values. Oral administration of NAC results in higher circulating concentrations of free Cys [10,11], yet without increasing the AUCs of both total cysteine and total glutathione [10]. The results of the present study are supportive of this observation. A likely not yet widely considered explanation for the increase of free Cys is formation of Cys through thiol/disulfide exchange reactions between circulating NAC and physiologic cystine and mixed cysteine. Our observations from isolated perfused liver experiments, with a calculated liver clearance of 0.11 mL^{-1} min^{-1} (Fig. 4), cast doubts on the real entity of the first-pass metabolism of NAC and as the main cause of its low bioavailability. By using ^{35}S-labeled NAC and by counting radioactivity, it was found that NAC absorption is complete [37]. It is therefore possible that absorbed ^{35}S-labeled NAC is extensively transformed into ^{35}S-labeled species, notably inorganic sulfate and taurine, in the gut.

The pharmacokinetics of NACET (Fig. 1) differs clearly from that of NAC. NACET is rapidly absorbed after oral administration but NACET concentration in plasma does not reach the plasma concentration of NAC upon oral administration of an equivalent NAC dose. This finding suggests that NACET rapidly enters the cells where it is "trapped". Indeed, after i.v. bolus the largest fraction of NACET is found in the cells of different tissues, including brain, mainly as NAC and Cys (Table 2). Inside the cells, NACET is rapidly de-esterified to NAC, most likely by the action of esterases, which is subsequently de-acetylated to cysteine (Fig. 1). We assume that Cys is slowly utilized in the synthesis of GSH and/or exported. Like liver, kidney and some other organs can further metabolize Cys to taurine and sulfate, in particular when Cys is in excess over the tissue-specific content [38].

Chronic treatment of rats with equivalent doses of NAC or NACET (Table 4) revealed that only NACET was able to increase significantly the GSH content of most tissues examined, even of the brain. This is of particular importance as it demonstrates that NACET crosses the blood brain barrier (see also Table 2). As alternatives to NAC, several other Cys precursors such as OTC have been used to enhance GSH in tissues, brain included. However, it is worth mentioning that OTC is converted to Cys by 5-oxoprolinase and some cells including neurons are lacking 5-oxoprolinase activity [39].

NAC is currently the antidote of choice for treating paracetamol overdose intoxication. In a rat model, our results confirm the utility of NAC as a paracetamol antidote (Table 5). Yet, in this model NACET was found to be more effective. The most accepted explanation for the protective role of Cys-containing drugs is that they can serve as a source of Cys required for synthesis of GSH, and the conjugation of GSH to NAPQI is enzymatically catalyzed before this reactive metabolite can damage the liver [40]. More recently, it has been proposed that NAC can directly act as a scavenger of ROS and can support mitochondrial energy metabolism [41]. Since NACET largely increases intracellular NAC and Cys, these two molecules can contribute to direct chemical NAPQI detoxification, besides serving as GSH precursors for enzymic GSH conjugation.

Unlike NAC, NACET passes rapidly the RBC membrane, yet inside RBCs no authentic NACET is detectable. NAC is the major NACET metabolite produced in RBCs and seems to accumulate therein (Fig. 6), obviously due to a low *N*-de-acetylation activity in human RBCs [42]. NACET-derived NAC is a potent protector against hydroperoxide-induced oxidative damage (Fig. 7). It is noteworthy that, in rat erythrocytes, NACET also entered the cell membrane

and was rapidly transformed into NAC. However, NAC was further de-acetylated to Cys, which was subsequently exported (not shown), suggesting a higher *N*-de-acetylation activity in rat RBCs compared to human RBCs. This is supported by findings showing that the SH group of $Cys^{\beta\text{-}125}$ in rat hemoglobin is very reactive [17].

Several approaches have been done to improve the quality and efficacy of stored RBCs. In particular, made efforts were focused on reducing oxidative damage by removing oxygen at the beginning of storage [43] and by increasing the antioxidant defense capacity of RBCs [44]. For instance, addition of GSH precursor amino acids and of NAC to stored RBCs has been proposed [44] in order to maintain a reducing environment in RBC cytosol. Based on the unique feature of NACET to accumulate in RBCs, one may speculate that "loading" RBCs with NACET may increase the life of banked RBCs. Autoxidation of hemoglobin is associated with production of superoxide, methemoglobin and finally denaturation to hemichromes, which then precipitate onto the lipids and the cytoskeleton of RBC membrane. Subsequent clustering of band 3 caused by such a precipitation may allow the recipient's immune system to recognize and cull the transfused RBCs from the circulation [45]. Treatment of RBCs with NACET may be an alternative approach in improving the quality of stored RBCs especially by increasing and maintaining their antioxidant capacity.

Our study shows that the main feature of NACET is its ability to enter cells and to produce NAC and Cys. Yet, NACET is also an H_2S producer (Fig. 5). H_2S is an important signaling gaseous molecule produced intra-cellularly from homocysteine and/or cysteine [24]. Due to the great interest in H_2S and the difficulty of synthesizing suitable H_2S-producing substances, NACET appears a good candidate for the oral use as an H_2S producer, with clear advantages over NAC.

5. Conclusions and perspectives

There is a widespread belief that increasing the cellular concentration of GSH is an easy task, safe and profitable for health. Our study shows that NACET possesses clearly improved pharmacokinetic properties compared to NAC, which is the most widely used GSH precursor drug. Thus, NACET holds the potential to be a more effective GSH precursor. Given the sophisticated mechanisms of the body to finely and potently regulate the thiol state intra- and extra-cellularly, we think that enhancement of the body's thiol store is challenging, not least because "thiols-overloaded" cells are likely to represent an unnatural condition with unforeseeable consequences for the whole organism. Nevertheless, as the most abundant redox buffer in the cell GSH is assumed to plays an important role in controlling biological stages of cells [46]. Consequently, increasing GSH in stressed cells may be effective by favoring all the processes promoted by a reductive environment.

The potential of NACET to substitute NAC as a mucolytic agent, as a paracetamol antidote and as a GSH-related antioxidant steams from our data, but its effects, either favorable or noxious, remain to be investigated more in depth in long term studies.

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