

Carbocisteine Reduces the Cytotoxicity of Oxaliplatin

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Z. Naturforsch. **67c**, 215–221 (2012); received May 24/November 25, 2011

Hepatic injury induced by oxaliplatin has been reported. Even though agents are available that reduce oxaliplatin-induced hepatocyte toxicity, their mode of action has remained obscure. In the present study, hepatic L02 cells were incubated with different combinations of oxaliplatin and carbocisteine. Significantly increased levels of reactive oxygen species (ROS) were found in L02 cells treated with oxaliplatin. Using 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium (MTS) as an indicator of cell viability and flow cytometry, we found that carbocisteine could reverse oxaliplatin-induced apoptosis of L02 cells. Western blot analysis demonstrated that oxaliplatin could induce apoptosis of L02 cells by reducing the Bcl-2/Bim ratio, stimulating the cytochrome c release, and activating caspase-3. All of these effects could be suppressed by carbocisteine. We further found that carbocisteine did not affect the anticancer effect of oxaliplatin against HT-29 cells. This is the first report opening prospects for the clinical use of carbocisteine in the pretreatment against liver injury accompanying the chemotherapy regimen with oxaliplatin.

Key words: Carbocisteine, Oxaliplatin, Hepatic Injury

Introduction

Oxaliplatin, a third-generation platinum analogue, like all platinum drugs, exerts its anticancer effects by forming inter- and intrastrand DNA adducts or cross-links, thereby inhibiting DNA replication and transcription. Although no increase in the morbidity and mortality rates was observed, liver injuries, including macroscopic blue liver, microscopic sinusoidal obstruction, and a significant decrease in liver function, were found in patients treated with oxaliplatin (Komori *et al.*, 2010; Rubbia-Brandt *et al.*, 2004; Synold *et al.*, 2007). These observations were supported by a multitude of other reports (Julie *et al.*, 2007; Kandutsch *et al.*, 2008; Mehta *et al.*, 2008; Pawlik *et al.*, 2007; Tisman *et al.*, 2004; Vauthey *et al.*, 2006). Because of these side effects, research on lowering oxaliplatin-induced hepatocyte toxicity is an important research topic.

Recent studies have shown that melatonin can attenuate oxaliplatin-induced apoptosis in renal carcinoma Caki cells by reversing reduced glutathione (GSH) depletion and Mcl-1 down-regulation (Um and Kwon, 2010), and ursodeoxycholic acid switches oxaliplatin-induced necrosis to apoptosis in HepG2 cells (Lim *et al.*, 2010). However, these reports dealt with the reduction of the toxicity of oxaliplatin on cancer cells. The mechanism underlying protective effects of certain agents against oxaliplatin-induced hepatocyte toxicity remained uncertain.

Carbocisteine treatment can prevent acute exacerbations in Chinese patients with chronic obstructive pulmonary disease (COPD) (Zheng *et al.*, 2008) and may inhibit cell damage induced by H₂O₂ in cultured human tracheal epithelial cells (Yoshida *et al.*, 2009). Given the fact that *N*-acetylcysteine (NAC) might reduce platinum-based chemotherapeutics-induced nephrotoxicity and provide otoprotection (Dickey *et al.*, 2005), we hypothesized that carbocisteine, a structural analogue of NAC (Fig. 1), might protect against

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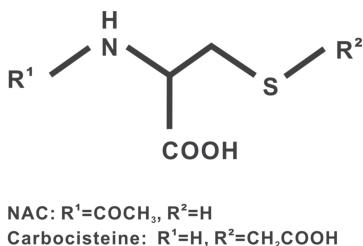


Fig. 1. Chemical structures of NAC and carbocisteine.

oxaliplatin-induced hepatocyte toxicity. The hypothesis was confirmed and we investigated the mechanism(s) of its action.

Material and Methods

Materials

All chemicals and solvents were from Sigma (St. Louis, MO, USA) and of analytical grade unless otherwise stated. RPMI-1640 medium and fetal calf serum were purchased from GIBCO/BRL (Grand Island, NY, USA). Monoclonal antibodies against human GAPDH, Bcl-2, Bax, Bim, caspase-3, cytochrome c, and horseradish peroxidase (HRP)-labeled second antibodies were the products of Cell Signaling Technology (Danvers, MA, USA).

Cell culture

Hepatic L02 cells were cultured at 37 °C, in a 5% CO₂ atmosphere, in RPMI-1640 medium containing 10% fetal calf serum, 1% penicillin (100 IU/mL), and 1% streptomycin (100 µg/mL). L02 cells were divided into 6 groups treated with different combinations of oxaliplatin (OX) and carbocisteine (CC) for 24 h unless otherwise stated: group 1 (control group), blank medium only; group 2, 40 µg/mL CC; group 3, 8 µg/mL OX; group 4, 40 µg/mL CC and 8 µg/mL OX; group 5, 1 µg/mL OX; group 6, 40 µg/mL CC and 1 µg/mL OX.

Cell viability

Cell viability was determined with the MTS assay (CellTiter 96® AQueous One Solution Cell Proliferation Assay; Promega Corporation, Madison, WI, USA).

Measurement of ROS generation

2',7'-Dichlorfluorescein-diacetate (DCFH-DA) was used to detect intracellular generation of

reactive oxygen species (ROS) by oxaliplatin as described previously (Zhai *et al.*, 2008).

Flow cytometric analysis of apoptosis

After apoptosis induction, L02 cells were collected with trypsin-EDTA, and apoptosis was evaluated using the Annexin-V-FLUOS staining kit (Roche Applied Science, Penzberg, Germany).

Western blotting

Twenty to 40 µg of protein from each sample were loaded on 5% stacking gels and separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), using 12% separating gels. Proteins were transferred onto nitrocellulose paper and blocked for 1 h with 5% skim milk in Tris-buffered saline (TBS) containing 0.01% NaN₃ and 0.1% Tween 20, and then incubated overnight with the primary antibody in TBS/0.1% Tween 20 at 4 °C. After three washes with TBS/0.1% Tween 20, membranes were incubated with HRP-conjugated secondary antibody at 1:5000 dilution for 1 h. Bands were visualized by enhanced chemiluminescence.

Determination of translocation by Western blotting

The cells were washed twice with ice-cold phosphate-buffered saline (PBS) and then resuspended in isotonic homogenization buffer (250 mM sucrose, 10 mM KCl, 1.5 mM MgCl₂, 1 mM EDTA-Na₂, 1 mM EGTA-Na₂, 1 mM dithiothreitol, 0.1 mM phenylmethylsulfonylfluoride, 10 µg/mL pepstatin A, 10 mM Tris-HCl, pH 7.4). After 80 strokes in a Dounce homogenizer, the unbroken cells were spun down at 30 x g for 10 min. The nuclei and mitochondria fractions were fractionated and removed at 6000 x g for 25 min. The supernatant was further subjected to centrifugation at 20000 x g for 60 min, and the final supernatant was used as the cytosolic fraction for the detection of released cytochrome c. Ten to 30 µg of protein from each sample were loaded on 5% stacking gels and separated by SDS-PAGE, using 12% separating gels, and then analysed as indicated above.

Statistical analyses

Results are expressed as means ± SD of six independent experiments, unless indicated otherwise. Statistical analyses were performed using Student's *t*-test as appropriate, with the level of significance set at *P* < 0.05.

Results

Effects of carbocisteine on oxaliplatin-induced loss of L02 cell viability

L02 cells were treated with oxaliplatin and carbocisteine for 24 h at the indicated concentrations. Absorbance at 490 nm decreased remarkably in a dose-dependent manner, as shown in Fig. 2A, suggesting that oxaliplatin significantly decreased the cell viability. This effect could be reversed by carbocisteine. Carbocisteine at the concentration of 40 µg/mL was maximally effective against oxaliplatin (Fig. 2B).

Effects of carbocisteine on oxaliplatin-induced apoptosis of L02 cells

Analysis of apoptosis in L02 cells was carried out by flow cytometry following oxaliplatin and carbocisteine treatment for 24 h. A significant increase in the percentage of apoptotic cells was detected in cells treated with oxaliplatin (Fig. 3B). At the same time, a significant decrease in the percentage of apoptotic cells was detected when carbocisteine was also present (Figs. 3A and 3C).

Effects of carbocisteine on oxaliplatin-induced changes in Bcl-2, Bax, and Bim protein levels

We performed Western blotting to check the levels of Bcl-2, Bax, and Bim. Levels of these three proteins were found down-regulated after oxaliplatin treatment for 24 h. When carbocisteine was also present, the levels were restored (Fig. 4). We then calculated the ratio of Bcl-2/Bax and Bcl-2/Bim. A decrease of the Bcl-2/Bim ratio was found in cells treated with oxaliplatin. This effect could also be reversed by carbocisteine (Figs. 4B and 4C).

Effects of carbocisteine on oxaliplatin-induced cytochrome c release and caspase-3 activation

We next investigated the expression levels of cytochrome c and caspase-3 by Western blotting. Cytochrome c release and the cleaved form of caspase-3 were detected in cells treated with oxaliplatin. These effects were suppressed by carbocisteine (Fig. 5).

Effects of carbocisteine on oxaliplatin-induced up-regulation of ROS level

Up-regulated ROS levels were detected in cells treated with oxaliplatin. This effect could also be reversed by carbocisteine (Fig. 6).

Effects of carbocisteine on the anticancer effect of oxaliplatin

We also tested the anticancer effect of oxaliplatin against HT-29 cells when carbocisteine was present (Table I). At the concentration of 40 µg/mL, carbocisteine could block the cytotoxic effect of oxaliplatin on L02 cells without affecting the antiproliferative effect on HT-29 cells.

Discussion

At anticancer effective dosage (Table I), oxaliplatin caused significant death of hepatocytes, which was evidenced by the MTS (Fig. 2) and flow cytometry assay (Fig. 3), respectively. The toxic effect of oxaliplatin was suppressed by carbocisteine (Figs. 2B, 3D, and 3F). To our knowledge, this is the first report on the protective effect of carbocisteine, an anti-COPD agent, against anticancer drug-induced hepatocyte toxicity.

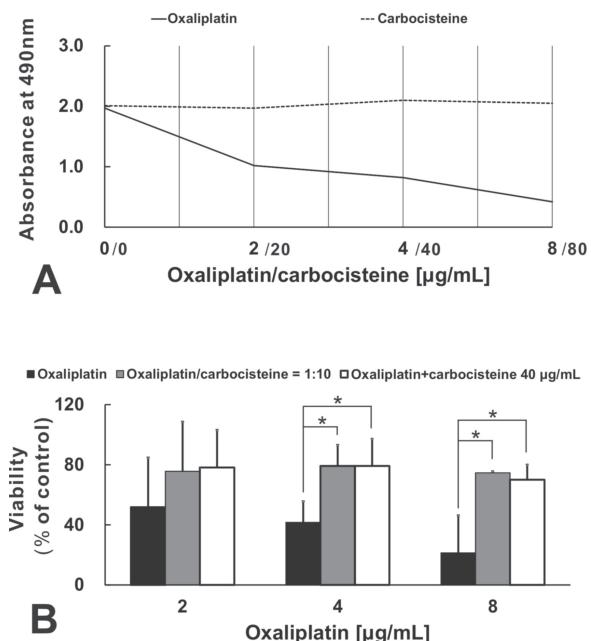


Fig. 2. Effects of oxaliplatin and carbocisteine on L02 cell proliferation. (A) Absorbance at 490 nm. L02 cells were divided into 4 groups which were treated with different combinations of oxaliplatin and carbocisteine as indicated by the (concentration of oxaliplatin)/(concentration of carbocisteine) ratios for 24 h. (B) Survival rates were calculated by dividing the absorbance of each group by that of the control group. Oxaliplatin was administered as indicated. Note that two different treatment strategies of carbocisteine were used as indicated by the insert. $n = 6$; * $P < 0.05$ versus control.

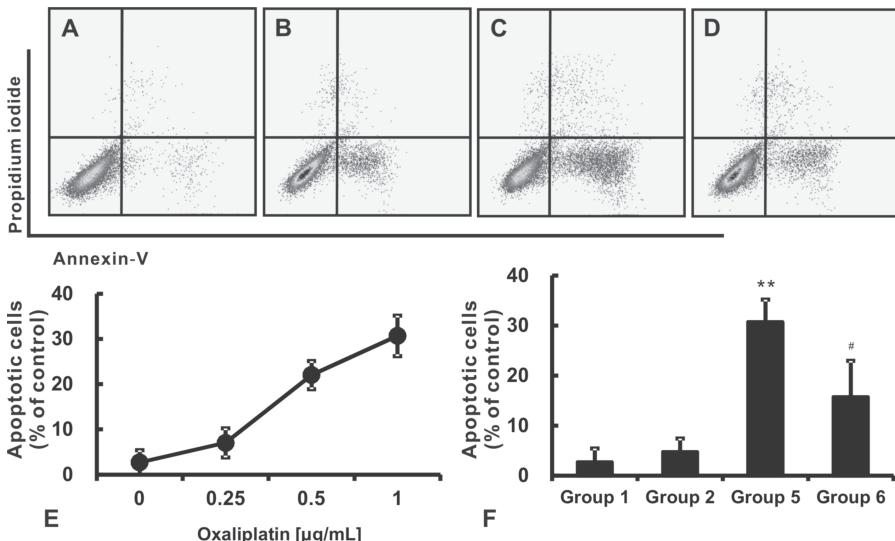


Fig. 3. Apoptotic effects of oxaliplatin (OX) and carbocisteine (CC) on L02 cells. (A)–(D) Cytometry assay of groups 1 (blank medium only), 2 (40 µg/mL CC), 5 (1 µg/mL OX), and 6 (40 µg/mL CC and 1 µg/mL OX). Representative results of 6 separate experiments. (E) Percentages of apoptosis after 24 h of treatment with various concentrations of oxaliplatin. (F) Statistical results from (A)–(D). $n = 6$; ** $P < 0.01$ versus control; # $P < 0.05$ versus group 5.

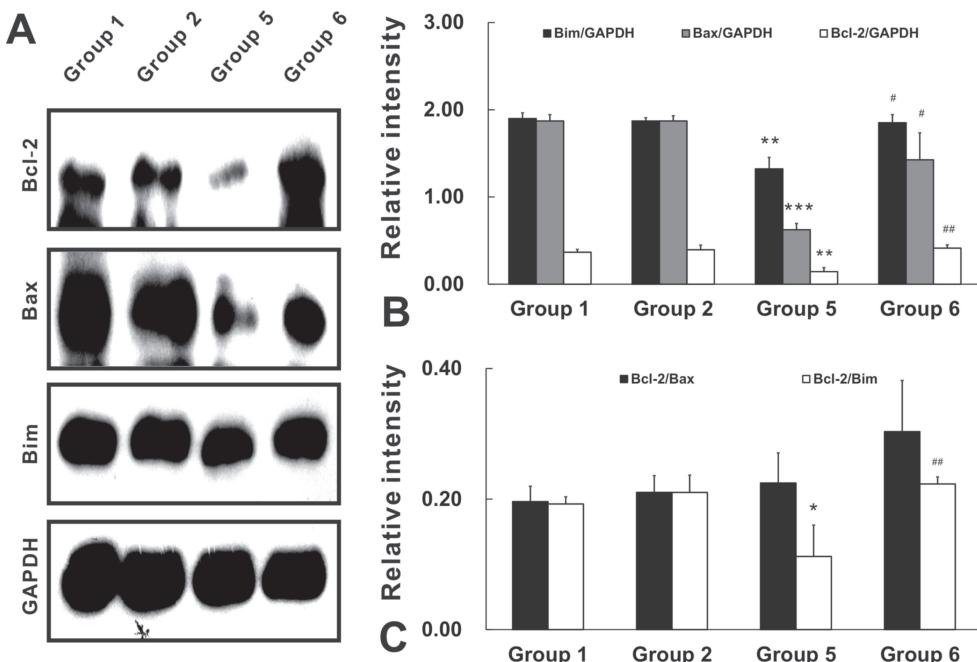


Fig. 4. Effects of oxaliplatin (OX) and carbocisteine (CC) on Bcl-2 family protein levels. Groups 1 (blank medium only), 2 (40 µg/mL CC), 5 (1 µg/mL OX), and 6 (40 µg/mL CC and 1 µg/mL OX) of cells were collected after 24 h of treatment. (A) Representative results of 6 separate experiments. (B) Results of scanning densitometry of the exposed films. Data are expressed as arbitrary units of intensity relative to internal standards (GAPDH). (C) Ratios of Bcl-2/Bax and Bcl-2/Bim were expressed as relative intensity. $n = 6$; * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.005$ versus control; # $P < 0.05$ and ## $P < 0.01$ versus group 5.

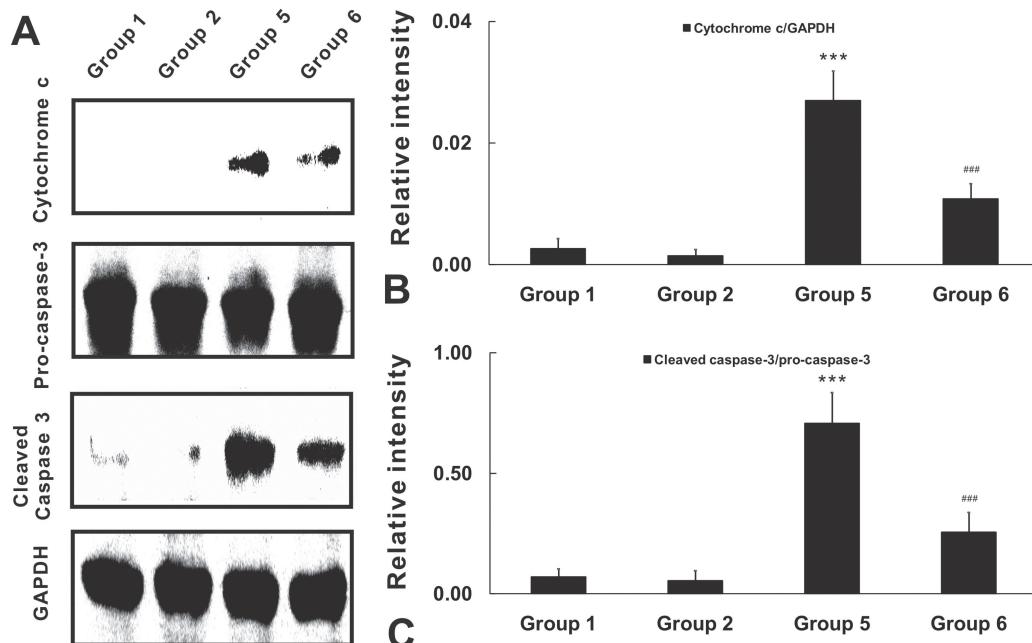


Fig. 5. Effects of oxaliplatin (OX) and carbocisteine (CC) on cytochrome c release and caspase-3 activation. Groups 1 (blank medium only), 2 (40 µg/mL CC), 5 (1 µg/mL OX), and 6 (40 µg/mL CC and 1 µg/mL OX) of cells were collected after 24 h of treatment. (A) Representative results of 6 separate experiments. (B, C) Results of scanning densitometry of the exposed films. Data are expressed as arbitrary units of intensity relative to internal standards (GAPDH). $n = 6$; *** $P < 0.005$ versus control; ## $P < 0.005$ versus group 5.

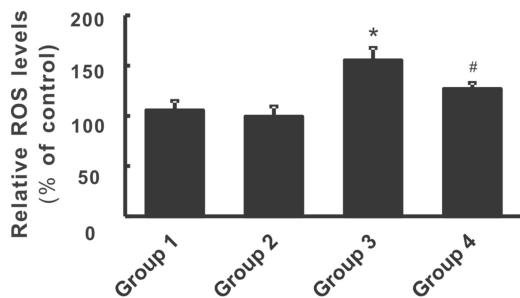


Fig. 6. Effects of oxaliplatin (OX) and carbocisteine (CC) on relative ROS levels. Group 1 (blank medium only), group 2 (40 µg/mL CC), group 3 (8 µg/mL OX), and group 4 (40 µg/mL CC and 8 µg/mL OX) of cells were collected after 24 h of treatment. $n = 6$; * $P < 0.05$ versus control; # $P < 0.05$ versus group 2.

Table I. Viability of HT-29 and L02 cells treated with oxaliplatin and carbocisteine.

Cell line	1 µg/mL oxaliplatin, no carbocisteine	1 µg/mL oxaliplatin, 40 µg/mL carbocisteine	P value
HT-29	40.34 ± 0.45	41.09 ± 0.65	0.5412
L02	71.77 ± 6.89	99.54 ± 8.10	0.0087

In fact, during oxaliplatin-induced apoptosis, suppression of Bcl-2 was observed in androgen-independent prostate cancer (AIPC) cells (Wilson *et al.*, 2008). Along with the down-regulation of Bcl-2, an up-regulation of Bax was also demonstrated in oxaliplatin-induced apoptosis of HCCM3 and Hep3B cells (Wang *et al.*, 2009). Evasion of apoptosis is commonly due to the overexpression of antiapoptotic proteins such as Bcl-2, which bind to the BH3 α-helical domain of pro-apoptotic proteins such as Bax and Bim, and thereby inhibit their functions. It seems that it is the balance between Bcl-2 and Bax (Bim) which mediates oxaliplatin-induced apoptosis. Because of this, we investigated the effect of carbocisteine on the levels of Bcl-2, Bax, and Bim.

However in the present study, during oxaliplatin-induced L02 cells apoptosis, not only a down-regulated level of Bcl-2 but also of Bax and Bim were observed (Figs. 4A and 4B). We found that co-administration of carbocisteine resulted in increased levels of Bcl-2, Bax, and Bim (Fig. 4). Furthermore, we checked the ratio of Bcl-2/Bax and Bcl-2/Bim and found the ratio of Bcl-2/Bax

not significantly affected by carbocisteine, while an obvious decline of the ratio of Bcl-2/Bim was observed in cultures treated with oxaliplatin. The decline was blocked by carbocisteine (Fig. 4C). This was consistent with the observed protective role of carbocisteine against oxaliplatin hepatotoxicity.

Bim was found to induce the cytochrome c release, a hallmark of type II apoptosis (Liu *et al.*, 2007; Schmich *et al.*, 2011). Blocking of endoplasmic reticulum stress-associated increases of Bim led to the inhibition of lipoapoptosis (Akazawa *et al.*, 2010). Hence, together with data presented in this report, the ratio of Bcl-2/Bim might be a favourable pro-apoptotic predictor for drug response and survival. The caspase-3-Bim axis is important in regulating both apoptosis and activation of osteoclasts (Tanaka *et al.*, 2010). A negative feedback loop in the caspase-3-Bim axis was established (Wakeyama *et al.*, 2007). These effects were confirmed in our present study, *i.e.* the expression levels of Bim and caspase-3 changed in opposite direction upon oxaliplatin stimulation (Fig. 4B versus Fig. 5B), and cytochrome c release was observed together with the down-regulation of Bim (Figs. 4A and 5A). The release of cytochrome c, down-regulation of Bcl-2, as well as a reduced ratio of Bcl-2/Bim suggested that oxaliplatin hepatotoxicity could result from mitochondrial dysfunction, which might lead to hepatic injury via oxidative stress (Kashimshetty *et al.*, 2009; Roy *et al.*, 2009).

We found that the levels of ROS significantly increased upon oxaliplatin stimulation, while an antioxidative effect of carbocisteine was observed (Fig. 6). However, it is believed that in addition to causing genomic instability, ROS can also increase tumour genesis by activating signalling pathways that regulate cellular proliferation, angiogenesis, and metastasis (Weinberg and Chadel 2009). Exogenous GSH did not change the inhibitory effects of oxaliplatin on A549 proliferation (Xu *et al.*, 2010). So we further checked the influence of carbocisteine on oxaliplatin cytotoxicity to HT-29 cells. Our data suggest that carbocisteine did not affect the anticancer effect of oxaliplatin, while it did reduce its hepatocyte toxicity (Table I).

Collectively, significant hepatocyte death caused by oxaliplatin, at anticancer effective dosage (Table I), can be suppressed by carbocisteine. This is the first report providing evidence for the clinical application of carbocisteine in pre-treatment against liver injury accompanying the chemotherapy regimen with oxaliplatin. The underlying mechanism involves imbalanced expression levels of Bcl-2 and Bim, cytochrome c release, caspase-3 activation, and increased ROS levels. Other effectors in oxaliplatin-induced apoptosis, such as p53 (Wang *et al.*, 2006), p38 MAPK (Fujie *et al.*, 2005), and survivin (Fujie *et al.*, 2005; Ngan *et al.*, 2008), might also play important roles in the protective effect of carbocisteine against oxaliplatin-induced hepatic injury, and the respective mechanisms deserve further investigation.

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