Available online at www.sciencedirect.com
SCIENCE DIRECT®
European Journal of Pharmacology 476 (2003) 185–191
www.elsevier.com/locate/ejphar

Ion dependence of cytotoxicity of carmustine against PC12 cells

Nina Doroshenko, Petro Doroshenko*

Ottawa Health Research Institute, 725 Parkdale Avenue, Ottawa, Ontario, Canada K1Y 4E9

Received 5 June 2003; received in revised form 24 July 2003; accepted 29 July 2003

Abstract

Cytotoxicity is a major complication of carmustine (1,3-bis(2-chloroethyl)-1-nitrosourea, BCNU) therapy for treatment of brain tumors and lymphomas. Using the lactate dehydrogenase (LDH) cell death assay in PC12 cells, we studied the role in this phenomenon of transmembrane ion fluxes that could be activated following inhibition by carmustine of glutathione reductase. The cytotoxic effect of carmustine developed during 4–6 h, with the EC_{50} of 27 μM. It depended on the extracellular Ca^{2+} concentration and substantially decreased upon Ca^{2+} removal. An almost complete suppression of toxicity was achieved when, additionally, monovalent cations were also replaced with impermeant organic cations. A similar loss of toxicity occurred in the presence of Ca^{2+} when extracellular Cl^- was replaced with impermeable gluconate. Various blockers of cation and Cl^- channels, as well as antioxidants also protected cells from carmustine. We conclude that carmustine toxicity against PC12 cells requires an influx of Ca^{2+} ions, supposedly through redox-sensitive cation channels.

Keywords: Nitrosourea; Glutathione reductase; Oxidative stress; Cation channel; Cl^- channel; Ca^{2+}

1. Introduction

Carmustine belongs to the group of DNA-alkylating and DNA-cross-linking agents, widely used as a cytostatic drug with activity against brain tumors, lymphomas, leukemia, malignant melanoma, and other neoplasms. It is generally accepted that the mechanism of action of carmustine is the formation of DNA interstrand cross-links (Colvin et al., 1976; Erickson et al., 1980; Gombar et al., 1980). The primary limiting complication of carmustine therapy is cytotoxicity, in the gastrointestinal system but particularly in the lung (Weiss et al., 1981). Proposed mechanisms of carmustine-induced cytotoxicity include DNA damage, adverse inflammation and an immune response, and glutathione depletion (Martin, 1997).

Depletion of cellular glutathione, a tripeptide which occurs in virtually all animal cells in relatively high (0.1–10 mM) concentrations, may result from carmustine-induced inhibition of a key enzyme in glutathione metabolism, glutathione reductase (Frischer and Ahmad, 1977). The reduced glutathione, GSH, serves as an antioxidant by reacting directly with free radicals and by providing substrate for the GSH peroxidases and the GSH transhydrogenases, with the resultant formation of oxidized glutathione, glutathione disulfide or GSSG. Regeneration of GSSG to GSH, catalyzed by glutathione reductase, together with de novo synthesis of GSH by the sequential action of γ-glutamylcysteine synthetase and GSH synthetase, maintain the reduced intracellular milieu (Meister, 1995). Inhibition of glutathione reductase by carmustine have been observed in all tested tissues, that is, in normal lung, liver, kidney, bone marrow, brain, and blood cells as well as in a number of tumor cells (e.g., Becker and Schirmer, 1995). In many of them, the activity of glutathione reductase is a rate-limiting step in redox regulation and inhibition of the enzyme by carmustine has been shown to deplete GSH and buildup GSSG (Smith and Boyd, 1984). Such a shift in redox status can substantially increase the permeability of plasma membrane to Ca^{2+} and Na^+ (e.g., Herson et al., 1999; Koliwad et al., 1996), leading to subsequent cell death (e.g., Hara et al., 2002; Mukherjee et al., 2002).

In the present study, we tested the hypothesis that carmustine toxicity involves Ca^{2+}/cation influx by investigating the dependence of carmustine-induced death of PC12 cells on ion composition of the extracellular medium. The results obtained support this hypothesis and are consistent with the involvement of redox-sensitive cation channels.

* Corresponding author. Tel.: +1-613-798-5555x16874; fax: +1-613-761-5330.
E-mail address: PDoroshenko@ohri.ca (P. Doroshenko).

0014-2999/$ - see front matter © 2003 Elsevier B.V. All rights reserved.
doi:10.1016/S0014-2999(03)02191-5

2. Materials and methods

2.1. Cell death assay

PC12 cells were seeded in 24-well plates at $\sim 2 \times 10^4$ cells/well and grown for 24 h in RPMI-1640 medium supplemented with 10% fetal bovine serum, 5% heat-inactivated horse serum and antibiotics. Carmustine-induced cell death was quantitatively assessed by measuring the activity of lactate dehydrogenase (LDH), released from damaged cells into the cell culture supernatant, using the LDH assay kit (Roche Molecular Biochemicals). As carmustine exposure lasted not more than 6 h, no significant changes in the number of cells in control and test wells were expected that would have complicated the use of the LDH cell death assay. In short, all wells on a plate were washed twice with a control solution before triplicate well groups were filled one each with the control solution and 0.2% Triton X-100 in the control solution, and the rest—with various test solutions (i.e., the control solution containing carmustine and/or other tested agents). Following treatment, during which the plates were kept in a CO_2 incubator at 37 °C, the extracellular medium was collected separately from each well and centrifuged for 10 min at $250 \times g$ to remove cell debris. Aliquots (100 μl) of the medium were then combined with equal volumes of the LDH assay mixture in a 96-well assay plate and incubated for 30 min at room temperature. The reaction was stopped by addition of 1 M HCl. LDH activity was assessed as a difference in absorbance at 492 and 690 nm using a microplate reader (MRX, Dynatech Laboratories). The fractions of dead cells in the test wells (six triplicate groups) were calculated as percentages of the difference in absorbance between the Triton wells (100% cell death) and the control wells (0% cell death). Since the LDH assay is performed on samples containing test substances, the lack of their direct effects on LDH activity was verified routinely by inclusion of these substances in control wells and sometimes also together with Triton.

2.2. Solutions

Solutions used in the studies on Ca^{2+}-dependence of carmustine toxicity contained (mM): $MgSO_4$ 0.8, KCl 5.36, KH_2PO_4 0.44, $NaHCO_3$ 4.17, NaCl 140, Na_2HPO_4 0.34, glucose 12.5, HEPES 10, pH 7.4, with addition of 0, 1.26, or 5 mM $CaCl_2$. In the Ca^{2+}-free solutions, $CaCl_2$ was replaced with *N*-methyl-D-glucamine (NMDG). To further reduce free Ca^{2+} levels, in some experiments the Ca^{2+}-free solution was supplemented with 0.5 mM EGTA. The composition of the Na^+- and Na^+, K^+-free solutions was similar to the one containing 5 mM $CaCl_2$ (5-Ca^{2+}), with the monovalent cations replaced on an equimolar basis with NMDG. The Cl^--free solution was also based on the 5-Ca^{2+} one, with all Cl^- replaced with membrane-impermeable gluconate. The cation-free solution contained (mM): NMDG 140, glucose 12.5, HEPES 10, pH 7.4 adjusted with HCl. The osmolarity of all solutions was kept at 300–310 mOsm (with mannitol, if necessary).

2.3. Materials

Carmustine (1,3-bis(2-chloroethyl)-1-nitrosourea, BCNU), flufenamic acid (*N*-(3-[trifluoromethyl]phenyl)anthranilic acid), IAA-94 (indanyloxyacetic acid 94), niflumic acid (2-(3-[trifluoromethyl]anilino)nicotinic acid), NPPB (5-Nitro-2-(3-phenylpropylamino)benzoic acid), and phloretin (3-(4-hydroxyphenyl)-1-(2,4,6-trihydroxyphenyl)-1-propanone) were from Sigma, DIDS (4,4′ -diisocyanatostilbene-2,2′ -disulfonic acid, Na salt) was from Calbiochem. Stock solutions of carmustine (20 mM in H_2O), and of ion channel blockers (in H_2O or dimethyl sulfoxide) were kept aliquoted at −20 °C until use. Final concentrations of dimethyl sulfoxide in the test solutions did not exceed 0.2%.

2.4. Statistics

Values are means ± S.E.M. of the indicated numbers (at least three) of independent experiments performed in triplicate. The significance of differences between experimental data groups was determined using Student's *t*-tests (GraphPad Prism 3.03, GraphPad Software, San Diego, CA). A P value less than 0.05 was considered significant. The P values are shown in the figures as follows: $P < 0.01$ (*), $P < 0.001$ (**), $P < 0.0001$ (***).

3. Results

3.1. Characterization of carmustine toxicity in PC12 cells

In an initial series of experiments, we used visual microscopy to assess the effects of carmustine on PC12 cells. Exposure of cells to >10 μM carmustine resulted in profound changes in their appearance indicative of cell injury. The characteristic feature of these changes was the formation of membrane blebs of various shapes and sizes all over cell bodies. At 75 μM carmustine, blebs first appeared after about 1-h exposure and became widely spread during longer exposures (not shown). After 2–3 h of incubation in the presence of 75 μM carmustine, almost all exposed cells developed blebs, reflecting severe cell damage caused by the drug. Control cells in similar conditions, without being exposed to carmustine, largely preserved their initial appearances during the same periods of observation.

The time course of cell death caused by exposure of PC12 cells to 75 μM carmustine is shown in Fig. 1. Confirming the morphological observations, significant amounts of released LDH were detected only after 1–2-h-long exposures, whereas most of the exposed cells died within 4 to 6 h. The half-maximal LDH release occurred after about 2.5-h-long exposures to carmustine ($T_{50} = 158$ min). Based on these temporal parameters of carmustine-

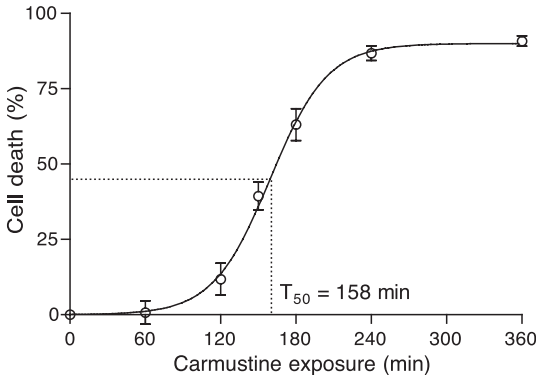

Fig. 1. Time course of carmustine cytotoxicity in PC12 cells. Cells were incubated with 75 μM carmustine in the 5-Ca^{2+} solution at 37 °C in a CO_2 incubator for various periods of time, up to 6 h. The fraction of cells killed by exposure to carmustine was determined immediately following the exposure. Each data point is a mean ± S.E.M. of three to six experiments (three wells/experiment). The fitted curve is a Boltzmann sigmoidal (r^2 = 0.9998), with the half-time T_{50} = 158 min.

induced cytotoxicity, in all the following experiments cells were exposed to the drug for a fixed period of 6 h.

We next determined the dose–response relationship for carmustine cytotoxicity. At concentrations < 10 μM, the drug's toxicity was very low. Above 10 μM, higher doses of carmustine killed progressively larger fractions of cells, with virtually all cells killed after being incubated for 6 h in the presence of 200 μM carmustine. The experimental data points representing the fraction of cells killed during 6-h-long exposures to different concentrations of carmustine were fit to a variable slope sigmoid (the Hill equation), with the EC_{50} value of 27 μM and n_H = 2.4 (Fig. 2).

3.2. Ion dependence of carmustine cytotoxicity

The main goal of the present study was testing the hypothesis that carmustine-induced oxidative stress may lead to activation of a cation-permeable conductance, thereby contributing to cell death. Since Ca^{2+} has been shown to be involved in many modes of cell death, including oxidative stress (reviewed in Ermak and Davies, 2002), we first investigated how carmustine cytotoxicity depends on extracellular Ca^{2+}. An increase in the extracellular Ca^{2+} concentration from 1.26 to 5 mM significantly enhanced the carmustine-induced death of PC12 cells (Fig. 3, bars marked 1.26 and 5-Ca). The fraction of cells killed by 75 μM carmustine in the 1.26-Ca^{2+} solution was 72 ± 4% of that in the 5-Ca^{2+} solution (N = 3). In the nominally Ca^{2+}-free solution, i.e. without added Ca^{2+} (Fig. 3, 0-Ca bar), this fraction further decreased to 35 ± 3% (N = 12). The effect of adding 0.5 mM EGTA to the Ca^{2+}-free solution (Fig. 3, EGTA bar) to remove residual Ca^{2+} was minor, with carmustine toxicity attenuated to 32 ± 1% (N = 3). The largest drop in the number of cells killed by carmustine, to 12 ± 3% (N = 8) of the 5-Ca^{2+} value, occurred upon replacement of all extracellular cations, both di- and monovalent, with impermeable NMDG (Fig. 3, 0-Cat bar). These latter observations suggested that in addition to Ca^{2+}, which obviously plays a central role in carmustine cytotoxicity, other extracellular cations, most likely Na^+ because of its abundance, also contributed to the cell death. Their contribution, however, was apparent only in the absence of Ca^{2+}. When almost all Na^+ (but 4.5 mM) or Na^+ and K^+ (Fig. 3, 0-Na and 0-Mono bar) were replaced with NMDG, while keeping 5 mM Ca^{2+} in the extracellular solution, carmustine toxicity was hardly affected: the fraction of dead cells was 92 ± 3% (N = 3) and 105 ± 8% (N = 6), respectively, of the value measured in the regular 5-Ca^{2+} solution. It appears, therefore, that the carmustine-induced cell death involves an influx of extracellular Ca^{2+} or, in its absence, of monovalent

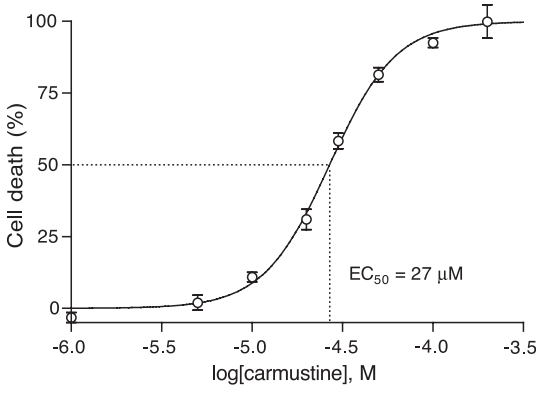

Fig. 2. Concentration-dependence of carmustine cytotoxicity in PC12 cells. Cells were exposed for a fixed period of 6 h to various concentrations of carmustine, from 1 to 200 μM, in the 5-Ca^{2+} solution. Each data point is a mean ± S.E.M. of three experiments. The fitted curve is a variable-slope sigmoidal (r^2 = 0.9996), with the EC_{50} = 27 μM and n_H = 2.4.

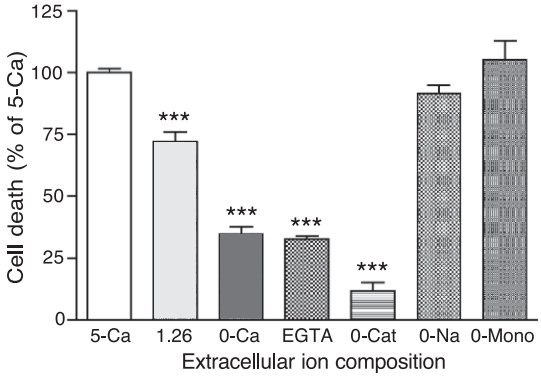

Fig. 3. Ion dependence of carmustine cytotoxicity. PC12 cells were incubated for 6 h in the presence of 75 μM carmustine in various solutions: with 5 mM Ca^{2+} (5-Ca bar), 1.26 mM Ca^{2+} (1.26 bar), nominally Ca^{2+}-free solution (0-Ca bar), Ca^{2+}-free solution with 0.5 mM EGTA (EGTA bar), cation-free solution (0-Cat bar), Na^+-free solution with 5 mM Ca^{2+} (0-Na bar), and Na^+ and K^+-free solution with 5 mM Ca^{2+} (Mono bar). Carmustine-induced cell death under each of the above conditions is presented as a percentage of that determined in the 5-Ca^{2+} solution in the same 24-well plate. Here and in the following figures, the data are shown as a mean ± S.E.M. (the number of triplicate experiments for each condition is given in the text).

cations. The influx supposedly occurs through the carmustine-induced cation channels.

3.3. Ion channel pharmacology of carmustine cytotoxicity

In an effort to substantiate the involvement of cation channels, we studied how various substances reported to block nonselective cation channels, such as flufenamic acid (Guinamard et al., 2002; Mukherjee et al., 2002; Weiser and Wienrich, 1996) and multivalent cations Co^{2+}, Ni^{2+}, Gd^{3+}, and La^{3+} (Herson et al., 1999; Hogg and Kozlowski, 2002; Hurne et al., 2002), affected carmustine cytotoxicity. In all experiments described below, the extracellular solutions contained 5 mM Ca^{2+} and the fraction of cells killed by carmustine in the absence of channel blockers was accepted as 100%. As shown in Fig. 4, in the presence of 200 μM flufenamic acid (Fig. 4, FFA bar) the fraction of cells killed by 75 μM carmustine was reduced more than 2-fold, to 45 ± 7% ($N=4$). Among the multivalent cations, the most effective cytoprotection was observed with 100 μM Co^{2+} (58 ± 7%, $N=3$), followed by 50 μM Gd^{3+} (70 ± 7%, $N=3$) and 100 μM La^{3+} ions (81 ± 6%, $N=3$). The neuroprotective effect of Ni^{2+}, at 100–300 μM, was not significant (at 300 μM: 88 ± 4%, $N=3$).

The conductive character of cation translocations underlying carmustine-induced cell death received further substantiation in experiments where external Cl^- was replaced with impermeable gluconate, while keeping the normal concentrations of Ca^{2+} and monovalent cations. The removal of extracellular Cl^- (Fig. 5, 0-Cl bar) had a cytoprotective effect equal to that of cation-free solution, with the cell death at only 14 ± 6% ($N=8$), indicating that the carmustine-induced influx of extracellular Ca^{2+}/cations requires the compensatory influx of their counter-ions, Cl^- ions, to maintain electroneutrality.

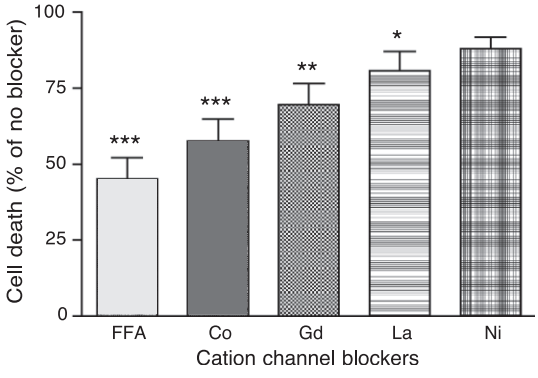

Fig. 4. Cytoprotection by cation channel blockers. PC12 cells were incubated for 6 h with 75 μM carmustine in the 5-Ca^{2+} solution in the presence of various blockers of cation channels: 200 μM flufenamic acid (FFA bar), 100 μM Co^{2+} (Co bar), 50 μM Gd^{3+} (Gd bar), 100 μM La^{3+} (La bar), and 100–300 μM Ni^{2+} ions (Ni bar). The fraction of dead cells under each of these conditions is shown as a percentage of that killed by 75 μM carmustine in the absence of channel blockers.

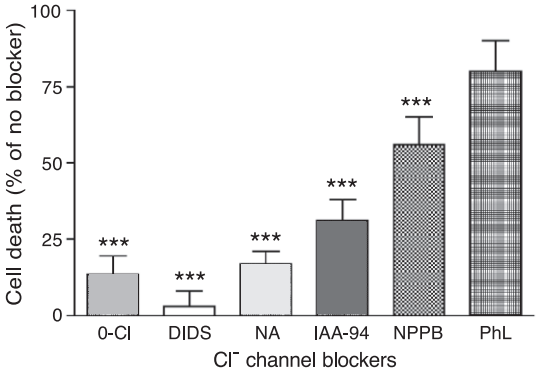

Fig. 5. Cytoprotection by Cl^- channel blockers. PC12 cells were incubated for 6 h with 75 μM carmustine in the 5-Ca^{2+} solution in the presence of various blockers of Cl^- channels: 50 μM DIDS (DIDS bar), 200 μM niflumic acid (NA bar), 100 μM IAA-94 (IAA-94 bar), 100 μM NPPB (NPPB bar), and 100 μM phloretin (Phl bar). Also shown is the fraction of cells killed by 75 μM carmustine in the Cl^--free solution (0-Cl bar) in the absence of Cl^- channel blockers. The fraction of dead cells under each of these conditions is shown as a percentage of that killed by 75 μM carmustine in the absence of channel blockers.

This suggestion is also consistent with the results of experiments with various Cl^- channel blockers, such as DIDS, IAA-94, NPPB, niflumic acid, and phloretin. The drugs were used in concentrations known to cause maximal effects on Cl^- channels. As shown in Fig. 5, 50 μM DIDS effectively prevented cell death caused by 75 μM carmustine: the fraction of dead cells was 3 ± 5% ($N=4$) of the value measured in the absence of Cl^- channel blockers. Niflumic acid (200 μM), IAA-94 (100 μM), and NPPB (100 μM) were also very efficient in suppressing carmustine cytotoxicity, with the fraction of dead cells measured 17 ± 4% ($N=7$), 31 ± 7% ($N=5$), and 56 ± 9% ($N=6$),

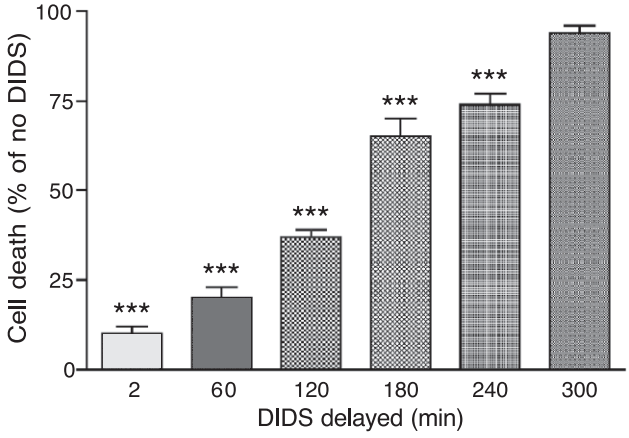

Fig. 6. Cytoprotection by delayed addition of DIDS. Cells in all test wells were exposed to 75 μM carmustine in the 5-Ca^{2+} solution for a fixed period of 6 h. Immediately after filling the wells with the test solution, 50 μM DIDS (as a 2 μl bolus of 7.5 mM DIDS per 300 μl solution in a well) was added to the first group of the test wells (2-min delay) and then, with a 1-h interval, to each of the remaining test well groups in sequence. The experiment was repeated three times. The fraction of dead cells under each of these conditions is shown as a percentage of that killed by 75 μM carmustine in the absence of DIDS.

respectively. Phloretin (100 μM) caused only a small decrease in the carmustine-induced cell death, to 80 ± 10% (N = 6).

Using DIDS as the most effective among the tested Cl^- channel blockers, we studied how its cytoprotective action was dependent on the timing of its addition in relation to the beginning of carmustine treatment. In these experiments, 50 μM DIDS were added to each of the carmustine-containing test wells with a varying delay, ranging from 0 to 5 h in 1-h intervals (Fig. 6). We found that even when added 4 h after the beginning of a 6-h-long exposure to carmustine, i.e. acting only during the last 2 h of the carmustine treatment, DIDS still significantly suppressed carmustine toxicity, to 74 ± 3% (N = 3) of the control value (no DIDS added). With shorter delays, the degree of suppression increased substantially, so that with a 1-h delay the fraction of cells killed by 75 μM carmustine in the presence of DIDS was only 20 ± 3% (N = 3) of the control.

3.4. Effects of antioxidants

Since by inhibiting glutathione reductase carmustine is expected to decrease the ratio of reduced to oxidized glutathione, thereby inducing oxidative stress conditions, we studied the effects on carmustine-induced cell death of several substances reported to have antioxidant properties: ellagic acid (Festa et al., 2001), deferoxamine (Regan and Rogers, 2003), and melatonin (Reiter, 1998). The antioxidants were added to the test wells 1 h prior to carmustine and were also present during the 6-h-long exposure to the drug. As shown in Fig. 7, all antioxidants protected PC12 cells from carmustine toxicity, albeit with different effectiveness. Both melatonin and deferoxamine each had a highly significant cytoprotective effect: the fraction of cells killed by 75 μM carmustine in their presence was reduced to 75 ± 5% (N = 3) for 250 μM melatonin and 35 ± 2% (N = 6) for 100 μM deferoxamine, compared with the carmustine-induced cell death in the absence of antioxidants. The least protective was ellagic acid, whose effect at 250 μM was mild and did not reach statistical significance: the fraction of cells killed was reduced only to 88 ± 6% (N = 3).

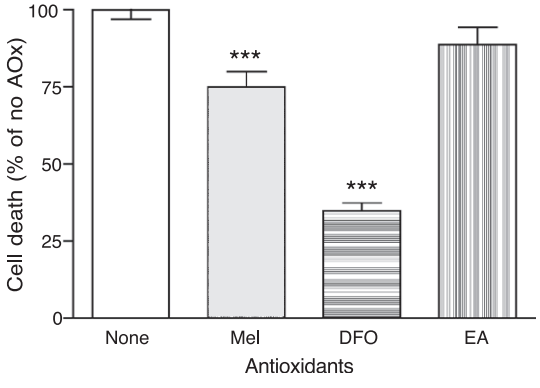

Fig. 7. Antioxidants protect PC12 cells against carmustine. Shown are the fraction of PC12 cells killed by exposure to 75 μM carmustine (for 6 h) in the absence (None bar) and in the presence of various antioxidants (AOx): 250 μM melatonin (Mel bar), 100 μM deferoxamine (DFO bar) and 250 μM ellagic acid (EA bar). The antioxidants were added 1 h before carmustine. Cell death in the carmustine-containing test wells without antioxidants is taken as 100%.

4. Discussion

The need to understand the mechanisms of carmustine cytotoxicity, which severely limits the use of more aggressive anticancer regiments, has encouraged studies of this phenomenon in a variety of cell types. The cell-related differences may underlie divergence of the reported toxicity of carmustine, with the half-maximal lethal dosage varying from 10 to 280 μM (Carmichael et al., 1988; Egyhazi et al., 1997; Heim et al., 2000; Khoshyomn et al., 2002; Ueda-Kawamitsu et al., 2002). The EC_{50} of 27 μM, derived from the experimental dose–response relationship for carmustine toxicity against PC12 cells (Fig. 2),which are widely used as a model for neuronal cells, falls well within the reported range. The steepness of the relationship, characterized by a larger than unity Hill coefficient n_H = 2.4 (see also Ueda-Kawamitsu et al., 2002), indicates a low toxicity of therapeutic dosages of carmustine only just smaller than the EC_{50}.

The time course of carmustine toxicity in PC12 cells bears a resemblance to an acute phase of carmustine-induced death of L1210 cells (Ueda-Kawamitsu et al., 2002): it developed with a delay of 1–1.5 h, followed by a massive loss of cells in the next 2–3 h, after which it flattened out (Fig. 1). It seems unlikely that this dynamics is affected by decomposition of carmustine inside cells and in the extracellular medium, reported to occur with the half-time of ~40 min (Weinkam and Deen, 1982; Ueda-Kawamitsu et al., 2002). Inhibition of glutathione reductase, that we hypothesize to be the major action of carmustine affecting cell viability in our experiments, has been shown to be irreversible and occurring quickly, with the half-time of about 10 min (Cohen and Duvel, 1988), i.e. long before the drug's decomposition.

The most important novel finding of this study is that cytotoxicity of carmustine depends on ion translocations across the cell membrane, primarily on Ca^{2+} influx. Arguably, the Ca^{2+} influx occurs via the carmustine-induced cation channels. The contribution of monovalent cations becomes apparent only under Ca^{2+}-free conditions (Fig. 3), and could be due to the change in selectivity of the putative channels in response to removal of the extracellular Ca^{2+} (e.g., Kostyuk and Krishtal, 1977; Tsien et al., 1987). The predominantly Ca^{2+}-selective permeability of the channels may explain the preservation of carmustine toxicity following withdrawal of monovalent cations while maintaining 5 mM Ca^{2+} in the extracellular medium (Fig. 3). Although the reverse mode of the Na^+/Ca^{2+} exchange in the absence of extracellular Na^+ (Blaustein and Lederer, 1999) could have enhanced Ca^{2+} influx and cell death, the close match of the

carmustine-induced cell death in the regular 5-Ca^{2+} and the Na^{+}-free solutions makes it a less likely possibility.

The suggestion that the Ca^{2+} influx contributing to carmustine toxicity occurs via cation channels is supported by the prominent cytoprotective effect of flufenamic acid (Fig. 4), a reputed blocker of cation channels (Guinamard et al., 2002; Meyer et al., 1996; Mukherjee et al., 2002; Weiser and Wienrich, 1996). A relatively poor cytoprotection by multivalent Co^{2+}, Gd^{3+}, and La^{3+} ions bears a resemblance to their low effectiveness in blocking hydrogen peroxide-activated nonselective cation channels in endothelial cells (Ji et al., 2002). The involvement of cation channels is consistent also with the cytoprotective effects of Cl^{-}-free extracellular solution and of the Cl^{-} channel blockers (Fig. 5), which suggest that the Ca^{2+} influx occurs down the electrochemical gradient and is accompanied by a compensatory influx of Cl^{-} ions. The observation that the Cl^{-} channel blocker suppressed carmustine-induced cell death even when added several hours after carmustine (Fig. 6) suggests that the putative cation channels stay open throughout carmustine exposure, thus providing a long-lasting influx of Ca^{2+} capable of causing cell injury.

The cytoprotective effect of the Cl^{-}-free solution may have an additional component related to Ca^{2+} chelation by gluconate, used to replace Cl^{-}. In the presence of $\sim$ 150 mM gluconate, from 5 mM Ca^{2+} added only $\sim$ 1.1 mM Ca^{2+} remain free, which is expected to lower carmustine toxicity slightly below that in the 1.26-Ca^{2+} solution, i.e. to $\sim$ 70% of its value in the regular 5-Ca^{2+} solution (Fig. 3). The observed cell death in the Cl^{-}-free solution was much lower, only 14% of the 5-Ca^{2+} value, confirming that the major part of the effect was due to reduction in the Cl^{-} influx.

A possible mechanism of activation of the putative cation channels may involve the oxidative stress resulting from inhibition by carmustine of glutathione reductase and subsequent depletion of GSH and accumulation of GSSG. This effect of carmustine has been found in every cell type tested (Becker and Schirmer, 1995) and presumably occurs also in PC12 cells. The cytoprotective effects of antioxidants observed in our study (Fig. 7) strongly support both the incidence of the oxidative stress and its role in carmustine toxicity. It is known that a shift in redox status can lead to activation of Ca^{2+}-permeable cation channels in the plasma membrane (e.g., Hara et al., 2002; Herson et al., 1999; Koliwad et al., 1996). In line with this mechanism are also reports that short exposures of endothelial cells to carmustine greatly potentiated the activation of nonselective cation channels by hydrogen peroxide (Koliwad et al., 1996) and increased the basal level of cytoplasmic Ca^{2+} (Elliott and Schilling, 1990).

In summary, the above data strongly suggest that a transmembrane influx of Ca^{2+}, supposedly through redox-sensitive cation channels, plays a significant role in carmustine toxicity in PC12 cells. Although the role of carmustine-induced glutathione reductase depletion and the consequences for glutathione metabolism and oxidative stress defense are likely to depend on the cell type, the prominence of the ionic component of carmustine toxicity in PC12 cells indicates its potential significance in other cell types as well.

Acknowledgements

Financial support was provided by grants from Ontario NeuroTrauma Foundation and Canadian Institutes of Health Research.

References

Becker, K., Schirmer, R.H., 1995. 1,3-Bis(2-chloroethyl)-1-nitrosourea as thiol-carbamoylating agent in biological systems. In: Parker, L. (Ed.), Methods in Enzymology, vol. 251. Academic Press, New York, NY, pp. 173–188.

Blaustein, M.P., Lederer, W.J., 1999. Sodium/calcium exchange: its physiological implications. Physiol. Rev. 79, 763–854.

Carmichael, J., Mitchell, J.B., DeGraff, W.G., Gamson, J., Gazdar, A.F., Johnson, B.E., Glatstein, E., Minna, J.D., 1988. Chemosensitivity testing of human lung cancer cell lines using the MTT assay. Br. J. Cancer 57, 540–547.

Cohen, M.B., Duvel, D.L., 1988. Characterization of the inhibition of glutathione reductase and the recovery of enzyme activity in exponentially growing murine leukemia (L1210) cells treated with 1,3-bis(2-chloroethyl)-1-nitrosourea. Biochem. Pharmacol. 37, 3317–3320.

Colvin, M., Brundrett, R.B., Cowens, W., Jardine, I., Ludlum, D.B., 1976. A chemical basis for the antitumor activity of chloroethylnitrosoureas. Biochem. Pharmacol. 25, 695–699.

Egyhazi, S., Edgren, M.R., Hansson, J., Krockel, D., Mannervik, B., Ringborg, U., 1997. Role of O^{6}-methylguanine-DNA methyltransferase, glutathione transferase M3-3 and glutathione in resistance to carmustine in a human non-small cell lung cancer cell line. Eur. J. Cancer 33, 447–452.

Elliott, S.J., Schilling, W.P., 1990. Carmustine augments the effects of *tert*-butyl hydroperoxide on calcium signaling in cultured pulmonary artery endothelial cells. J. Biol. Chem. 265, 103–107.

Erickson, L.C., Laurent, G., Sharkey, N.A., Kohn, K.W., 1980. DNA cross-linking and monoadduct repair in nitrosourea-treated human tumour cells. Nature 288, 727–729.

Ermak, G., Davies, K.J.A., 2002. Calcium and oxidative stress: from cell signaling to cell death. Mol. Immunol. 38, 713–721.

Festa, F., Aglitti, T., Duranti, G., Ricordy, R., Perticone, P., Cozzi, R., 2001. Strong antioxidant activity of ellagic acid in mammalian cells in vitro revealed by the comet assay. Anticancer Res. 21 (6A), 3903–3908.

Frischer, H., Ahmad, T., 1977. Severe generalized glutathione reductase deficiency after antitumor chemotherapy with BCNU [1,3-bis(chloroethyl)-1-nitrosourea]. J. Lab. Clin. Med. 89, 1080–1091.

Gombar, C.T., Tong, W.P., Ludlum, D.B., 1980. Mechanism of action of the nitrosoureas: IV. Reactions of BCNU and CCNU with DNA. Biochem. Pharmacol. 22, 2639–2643.

Guinamard, R., Rahmati, M., Lenfant, J., Bois, P., 2002. Characterization of a Ca^{2+}-activated nonselective cation channel during dedifferentiation of cultured rat ventricular cardiomyocytes. J. Membr. Biol. 188, 127–135.

Hara, Y., Wakamori, M., Ishii, M., Maeno, E., Nishida, M., Yoshida, T., Yamada, H., Shimizu, S., Mori, E., Kudoh, J., Shimizu, N., Kurose, H., Okada, Y., Imoto, K., Mori, Y., 2002. LTRPC2 Ca^{2+}-permeable channel activated by changes in redox status confers susceptibility to cell death. Mol. Cell 9, 163–173.

Heim, M.M., Eberhardt, W., Seeber, S., Muller, M.R., 2000. Differential modulation of chemosensitivity to alkylating agents and platinum com-

pounds by DNA repair modulators in human lung cancer cell lines. J. Cancer Res. Clin. Oncol. 126, 198–204.

Herson, P.S., Lee, K., Pinnock, R.D., Hughes, J., Ashford, M.L.J., 1999. Hydrogen peroxide induces intracellular calcium overload by activation of a nonselective cation channel in an insulin-secreting cell line. J. Biol. Chem. 274, 833–841.

Hogg, D.S., Kozlowski, R.Z., 2002. Hypoxia activates a background conductance in freshly isolated pulmonary arterial endothelial cells of the rat. FEBS Lett. 522, 125–129.

Hurne, A.M., Chai, C.L.L., Moerman, K., Waring, P., 2002. Influx of calcium through a redox-sensitive plasma membrane channel in thymocytes causes early necrotic cell death induced by the epipolythiodioxopiperazine toxins. J. Biol. Chem. 277, 31631–31638.

Ji, G., O'Brien, C.D., Feldman, M., Manevich, Y., Lim, P., Sun, J., Albelda, S.M., Kotlikoff, M.I., 2002. PECAM-1 (CD31) regulates a hydrogen peroxide-activated nonselective cation channel in endothelial cells. J. Cell Biol. 157, 173–184.

Khoshyomn, S., Nathan, D., Manske, G.C., Osler, T.M., Penar, P.L., 2002. Synergistic effect of genistein and BCNU on growth inhibition and cytotoxicity of glioblastoma cells. J. Neurooncol. 57, 193–200.

Koliwad, S.K., Elliott, S.J., Kunze, D.L., 1996. Oxidized glutathione mediates cation channel activation in calf vascular endothelial cells during oxidant stress. J. Physiol. 495, 37–49.

Kostyuk, P.G., Krishtal, O.A., 1977. Effects of calcium and calcium-chelating agents on the inward and outward currents in the membrane of mollusc neurones. J. Physiol. 270, 569–580.

Martin II, W.J., 1997. Injury from drugs. The Lung: Scientific Foundations. Lippincott-Raven, Philadelphia, PA, pp. 2466–2473.

Meister, A., 1995. Glutathione metabolism. In: Packer, L. (Ed.), Methods in Enzymology, vol. 251. Academic Press, New York, NY, pp. 3–7.

Meyer, T.N., Gloy, J., Hug, M.J., Greger, R., Schollmeyer, P., Pavenstadt, H., 1996. Hydrogen peroxide increases the intracellular calcium activity in rat mesangial cells in primary culture. Kidney Int. 49, 388–395.

Mukherjee, S.B., Das, M., Sudhandiran, G., Shaha, C., 2002. Increase in cytosolic Ca^{2+} levels through the activation of nonselective cation channels induced by oxidative stress causes mitochondrial depolarization leading to apoptosis-like death in *Leishmania donovani* promastigotes. J. Biol. Chem. 277, 24717–24727.

Regan, R.F., Rogers, B., 2003. Delayed treatment of hemoglobin neurotoxicity. J. Neurotrauma 20, 111–120.

Reiter, R.J., 1998. Oxidative damage in the central nervous system: protection by melatonin. Prog. Neurobiol. 56, 359–384.

Smith, A.C., Boyd, M.R., 1984. Preferential effects of 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU) on pulmonary glutathione reductase and glutathione/glutathione disulfide ratios: possible implications for lung toxicity. J. Pharmacol. Exp. Ther. 229, 658–663.

Tsien, R.W., Hess, P., McCleskey, E.W., Rosenberg, R.L., 1987. Calcium channels: mechanisms of selectivity, permeation, and block. Annu. Rev. Biophys. Biophys. Chem. 16, 265–290.

Ueda-Kawamitsu, H., Lawson, T.A., Gwilt, P.R., 2002. In vitro pharmacokinetics and pharmacodynamics of 1,3-bis(2-chloroethy)-1-nitrosourea (BCNU). Biochem. Pharmacol. 63, 1209–1218.

Weinkam, R.J., Deen, D.F., 1982. Quantitative dose–response relations for the cytotoxic activity of chloroethylnitrosoureas in cell culture. Cancer Res. 42, 1008–1014.

Weiser, T., Wienrich, M., 1996. Investigations on the mechanism of action of the antiproliferant and ion channel antagonist flufenamic acid. Naunyn-Schmiedeberg's Arch. Pharmacol. 353, 452–460.

Weiss, R.B., Poster, D.S., Penta, J.S., 1981. The nitrosoureas and pulmonary toxicity. Cancer Treat. Rev. 8, 111–125.