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Acrolein and chloroacetaldehyde: An examination of the cell and cell-free biomarkers of toxicity

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ABSTRACT

Cyclophosphamide and ifosfamide are two commonly used DNA-alkylating agents in cancer chemotherapy that undergo biotransformation to several toxic and non-toxic metabolites, including acrolein and chloroacetaldehyde (CAA). Acrolein and CAA toxicities occur by several different mechanisms, including ROS formation and protein damage (oxidation), however, these pathways of toxicity and protecting agents used to prevent them have yet to be compared and ranked in a single study. This research focused on the molecular targets of acrolein and CAA toxicities and strategies to decrease toxicities. Hepatocyte viability (cytotoxicity) was assessed using Trypan blue uptake; formation of reactive oxygen species (ROS) and endogenous H_2O_2 were also assessed in the hepatocyte model. In cell-free models (bovine serum albumin and hepatic microsomes), protein carbonylation was the measurement of toxicity. The present study demonstrated that acrolein was a more potent toxin than CAA for freshly isolated rat hepatocytes, bovine serum albumin and rat hepatic microsomes. Acrolein protein carbonylation was dependent on its concentration; as acrolein concentration increased, protein carbonylation increased in a linear trend, whereas, CAA deviated from the trend and did not cause protein carbonylation at lower concentrations (<400 μM). Aldehyde dehydrogenase (ALDH) is a major pathway for detoxifying pathway for CAA in hepatocytes, as a 3-fold increase in cytotoxicity occurred when cells were incubated with cyanamide, an ALDH inhibitor. Inhibiting ALDH or depleting GSH in hepatocytes increased cytotoxicity by about 3-fold in acrolein-treated hepatocytes. The overall effectiveness of protecting agents to prevent or suppress acrolein or CAA toxicities in cell and cell-free models were ranked in order of most effective to least effective: reducing agents (sodium borohydride, sodium bisulfite) > thiol-containing compounds (*N*-acetylcysteine, cysteine, glutathione, 2-mercaptoethane sulfonate [MESNA], penicillamine) > carbonyl scavengers/amines (aminoguanidine, hydralazine, hydroxylamine) > antioxidants/ROS scavengers (ascorbic acid, Trolox; only utilized in hepatocyte system). An understanding of acrolein and CAA toxicities and the ability of protecting agents to protect against toxicities may help to establish or improve existing therapeutic interventions against the side effects associated with acrolein or CAA in cyclophosphamide or ifosfamide treatment.

1. Introduction

Cyclophosphamide and ifosfamide are DNA-alkylating agents commonly used in cancer chemotherapy. Metabolism of these compounds mainly occurs in the liver, but can also occur in other sites, including erythrocytes, kidneys and the tumor itself. Although the metabolic pathways are similar, cyclophosphamide and ifosfamide differ in the degree of formation of certain metabolites. Both compounds are administered as prodrugs that undergo biotransformation catalyzed by cytochrome P450s. As a byproduct of this reaction, acrolein is produced [1]. Ifosfamide and, to a lesser extent, cyclophosphamide are also inactivated by *N*-dechloroethylation, resulting in *N*-dechloroethylated metabolites and the byproduct chloroacetaldehyde (CAA) [1,2]. Although these anticancer drugs are widely used for the treatment of a variety of tumor types, their use is associated with systemic toxicities resulting from their subsequent metabolism. Acrolein is known to be responsible for the hemorrhagic cystitis observed in some patients, whereas CAA is said to be responsible for nephrotoxicity and neurotoxicity. About 25–60% of ifosfamide is metabolized to CAA and its dechloroethylated metabolites that compete with the 4-hydroxylation pathway. In contrast, only about 10% of cyclophosphamide is dechloroethylated. This could explain the decreased neuro- and nephrotoxicity side effects of cyclophosphamide as compared to ifosfamide [1,3].

Acrolein is a highly reactive α,β-unsaturated aldehyde that readily reacts with cellular nucleophiles, such as the thiol groups

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of cysteine residues in proteins and glutathione (GSH), as well as nitrogen atoms in lysine and histidine groups [4]. Previous literature suggests that acrolein bladder toxicity can be explained in three steps: (1) acrolein rapidly enters uroepithelial cells where it activates intracellular reactive oxygen species (ROS) and NO production through iNOS activation, leading to (2) peroxynitrite production. Once peroxynitrite is formed, it can lead to (3) damage to protein, DNA and lipids [5]. Chloroacetaldehyde, like acrolein, causes GSH depletion at toxic concentrations (above 300 μM) and partial GSH depletion at sub-toxic concentrations (150–200 μM) in hepatocytes, followed by thiol protein adduct formation and finally results in lipid peroxidation and cytotoxicity [6]. These cytotoxic byproducts can be detoxified by various aldehyde dehydrogenases (ALDHs) to their corresponding and less toxic acids, as well as by conjugation with GSH [1]. Among the metabolic detoxification pathways, there are several pharmacological approaches to limit the toxicities of reactive carbonyl species (RCS). Direct trapping of these compounds, such as the formation of covalent adducts with nucleophilic thiols or direct Schiff bases adducts with nucleophilic nitrogens, plays an important role in the inhibition of toxicities, as well as increasing the antioxidant defense systems against oxidative stress. Compounds possessing these capabilities include thiols, carbonyl scavengers/amines, and reducing agents or antioxidants [7].

Acrolein and CAA toxicities occur by several different mechanisms, but extent of toxicity and protection by various chemicals need to be compared and ranked in a single study. These studies could provide a better understanding into the therapeutic value of protective agents currently being prescribed in combination with ifosfamide and cyclophosphamide treatment, and for other agents not currently being used, but which have demonstrated scavenging or protecting effects against acrolein or CAA or other toxic aldehydes.

2. Materials and methods

2.1. Chemicals

Type II Collagenase was purchased from Worthington (Lakewood, NJ). *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES) was purchased from Boehringer–Mannheim (Montreal, Canada). Acrolein, chloroacetaldehyde (CAA), trichloroacetic acid (TCA), dinitrophenylhydrazine (DNPH), (±)-6-hydroxy-2,5,7,8-tetramethylchromamane-2-carboxylic acid (Trolox), 2-mercaptoethanesulfonate (MESNA) and all other chemicals were obtained from Sigma–Aldrich Corp. (Oakville, ON, CAN).

2.2. Animal treatment and hepatocyte preparation

Male Sprague–Dawley rats weighing 275–300 g (Charles River Laboratories) were used according to the guidelines of the Canadian Council on Animal Care [8]. Hepatocytes were isolated from rats by collagenase perfusion of the liver as described by Moldéus and colleagues [9]. Isolated hepatocytes (10^6 cells/mL, 10 mL) were suspended in Krebs-Henseleit buffer containing 12.5 mM HEPES (free acid) with pH readjusted to 7.4, in rotating 50 mL round-bottomed flasks, under an atmosphere of 95% O_2 and 5% CO_2 or 1% O_2, 94% N_2 and 5% CO_2 in a water bath of 37 °C for 30 min prior to the addition of chemicals.

2.3. Cell viability

Hepatocyte viability was assessed microscopically by plasma membrane disruption as determined by the Trypan blue (0.1% w/v) exclusion test [9]. Hepatocyte viability was determined every 30 min during a 3 h incubation period. Hepatocytes used were 80–90% viable before use. GSH-depleted hepatocytes were obtained by preincubating hepatocytes with 200 μM 1-bromoheptane for 30 min prior to the addition of other agents [10]. Aldehyde dehydrogenase (ALDH)-inhibited hepatocytes were obtained by preincubating cells with 200 μM cyanamide for 45 min. The concentrations of inhibitors/modulators used were nontoxic.

2.4. Microsomal preparation

Adult male Sprague–Dawley rats (250–300 g) were anesthetized by sodium pentobarbital (60 mg/kg body). Livers were removed under sterile conditions and perfused with KCl solution (1.18% w/v, 4 °C). Hepatic microsomes were prepared by differential centrifugation as described by Dallner et al. [11]. The microsomal pellet was suspended and homogenized in sterile 50 mM potassium phosphate buffer with 0.23% (w/v) KCl pH 7.4 before storage at −80 °C. Microsomal protein was determined by the method of Joly et al. [12].

2.5. Protein carbonylation assay

Bovine serum albumin or microsome suspensions were either treated simultaneously with acrolein or CAA and the therapeutic agents, or the suspensions were pre-treated with acrolein or CAA for 1 h prior to the addition of the protecting agents for the antidotal effect. Time zero of the reaction began with the addition of acrolein or CAA. The total protein-bound carbonyl content was measured by derivatizing the protein carbonyl adducts with 2,4-dinitrophenylhydrazine (DNPH). An aliquot of hepatocytes, bovine serum albumin (2 mg/mL or 30 μM in 0.1 M sodium phosphate buffer, pH 7.4) or microsomal suspension (0.5 mL) at different time points was added to an equivalent volume (0.5 mL) of 0.1% DNPH (w/v) in 2.0 N HCl and allowed to incubate for 1 h at room temperature. The reaction was terminated and the total cellular protein precipitated by the addition of 1.0 mL volume of TCA (20% w/v). Protein was pelleted by centrifugation at 5000*g* for 1 min, and the supernatant was discarded. Excess unincorporated DNPH was extracted three times using an excess volume (0.5 mL) of ethanol:ethyl acetate (1:1) solution. Following extraction, the recovered cellular protein was dried under a stream of nitrogen and dissolved in 1 mL of 2 M Tris-buffered 8.0 M guanidine–HCl, pH 7.2. The resulting solubilized hydrazones formed were measured using a SpectraMax Plus384 spectrophotometer at 370 nm. The concentration of DNPH derivatized protein carbonyls dissolved in guanidine-HCl was determined using an extinction coefficient of 22,000 $M^{-1}cm^{-1}$ [13].

2.6. Reactive oxygen species (ROS) formation

Hepatocyte ROS formation was determined by adding dichlorofluorescein diacetate (DCFH-DA) to the hepatocyte incubate. DCFH-DA penetrates hepatocytes and is hydrolyzed to form a non-fluorescent dichlorofluorescein (DCF). DCF then reacts with 'ROS' to form the highly fluorescent dichlorofluorescein and effluxes the cell. ROS formation was assayed by withdrawing 1 mL samples, which were then centrifuged for 1 min at 5000*g*. The cells were resuspended in 1 mL Krebs-Heinseleit buffer containing 2 μM DCFH-DA and incubated at 37 °C for 10 min, and the fluorescence intensity was measured using a SpectraMax Gemini XS fluorimeter at 490 nm excitation and 520 nm emission [14].

2.7. Hepatocyte H_2O_2 measurement

H_2O_2 was measured in hepatocytes by taking aliquots at 30 or 90 min using the FOX 1 reagent (ferrous oxidation of xylenol